UNIVERSIDADE DE LISBOA

Faculdade de Farmácia

Research Institute for Medicines
(iMed.ULisboa)

Neuron Glia Biology in Health and Disease Group

Endothelial progenitor cells and associated pathways in multiple myeloma

Maria Margarida Batista Tenreiro

Dissertação de Mestrado para obtenção do grau de Mestre em Ciências Biofarmacêuticas

Orientadora: Prof.ª Doutora Maria Alexandra Brito

Co-orientadora: Prof.ª Doutora Maria Leonor Correia

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Abstract

Multiple myeloma (MM) is characterized by the clonal expansion of plasma cells (PCs) in the bone marrow (BM) that leads to bone destruction, anaemia and renal failure. Although there are several therapeutic options nowadays, there is still no effective cure and the standard survival up to 4 years. The evolution from the asymptomatic stage of monoclonal gammopathy of undetermined significance (MGUS) to MM and the progression of the disease itself are related with cellular and molecular alterations in the BM microenvironment, namely, the development of the vasculature. In postnatal vasculogenesis, there is stimulation of the recruitment of BM progenitors known as endothelial progenitor cells (EPCs) to the tumour vasculature, which will incorporate newly-forming blood vessels and differentiate into endothelial cells. The mobilization of EPCs is tightly controlled by cells and molecules in the BM microenvironment. With this retrospective study, we intended to evaluate the potential of EPCs as biomarkers for MM progression and response to therapy, while assessing their relationship with PCs and the signalling receptors C-X-C motif chemokine receptor (CXCR)4 and platelet-derived growth factor receptor (PDGFR)-β in sequentially collected BM smears from MM patients in different disease stages. Thus, we aimed to: 1) develop a method to quantify EPCs in BM smears from MM patients, 2) establish the temporal evolution of EPCs levels and verify their relationship with PCs, and 3) evaluate the content of $CXCR4^+$ and PDGFR-β^+ cells and their connection with EPCs. We examined the percentage of EPCs with multiple immunofluorescence, and with single immunofluorescence the percentage of $CXCR4^+$ and PDGFR-β^+ cells, from sequentially collected BM smears from followed MM patients in two main groups: i) patients that evolved from MGUS to MM, and ii) patients with MM that received treatment. Our results show that MM patients with higher BM PCs at diagnosis had significantly higher levels of BM EPCs in MGUS in comparison to the patients with lower MM PCs. On the other hand, MM patients who entered remission after treatment displayed lower initial levels of EPCs than MM patients who did not achieve remission. Both $CXCR4^+$ and PDGFR-β^+ cells levels were correlated with EPCs levels throughout the analysed stages, which suggests that these receptors may be involved in EPC-related molecular processes such as recruitment to the tumour location and proliferation in MM. Taken together, our findings highlight for the first time in sequential archived BM samples that EPCs can constitute a biomarker for an aggravated progression from MGUS to MM and a worse response to therapy. Moreover, we also underline the need to study the mechanisms related to EPC-mediated vasculogenesis in MM.

Keywords: endothelial progenitor cells, monoclonal gammopathy of undetermined significance, multiple myeloma, remission, vasculogenesis.

Resumo

O mieloma múltiplo (MM) é caracterizado pela expansão clonal de plasmócitos na medula óssea (MO) que leva à destruição do osso, anemia e insuficiência renal. Embora existam atualmente várias opções terapêuticas, não existe uma cura efetiva e o tempo médio de vida após diagnóstico é de 3 a 4 anos. A evolução do estado assintomático de gamopatia monoclonal de significado indeterminado (GMSI) para MM e a progressão da doença em si estão relacionadas com alterações celulares e moleculares dentro do microambiente da MO, nomeadamente com o desenvolvimento da vasculatura. Na vasculogénese pós-natal existe estimulação do recrutamento de progenitores da MO conhecidos como células endoteliais progenitoras (CEPs) para a vasculatura do tumor. As CEPs incorporam os vasos sanguíneos recém-formados e diferenciam-se em células endoteliais. A mobilização das CEPs é altamente controlada pelas células e moléculas no microambiente da MO. Este estudo retrospetivo teve como objetivo principal avaliar o potencial das CEPs como biomarcadores para a progressão de MM e resposta à terapia, assim como determinar a relação das CEPs com os plasmócitos e recetores de sinalização CXCR4 e recetor β do fator de crescimento derivado de plaquetas (PDGFR-β) em amostras colhidas sequencialmente de esfregaços de MO de pacientes com MM em várias fases da doença. Assim sendo, os objetivos específicos deste trabalho foram: 1) desenvolver um método de quantificação de CEPs em esfregaços de MO de pacientes com MM, 2) estabelecer a evolução temporal dos níveis de CEPs e verificar a sua relação com os plasmócitos, 3) avaliar o conteúdo de células positivas para os recetores CXCR4 e PDGFR-β e a sua ligação aos plasmócitos. Examinámos a percentagem de CEPs recorrendo a tripla imunofluorescência e, com imunofluorescência única, a percentagem de células $CXCR4^+$ e PDGFR-β^+, a partir de esfregaços de MO recolhidos sequencialmente de doentes com MM seguidos em dois grupos principais: i) doentes que evoluíram de GMSI para MM, e ii) doentes com MM que receberam tratamento. Os nossos resultados mostram que os doentes com MM com conteúdo de plasmócitos na MO mais elevados no momento do diagnóstico apresentaram níveis significativamente mais elevados de CEPs na MO em GMSI em comparação com os pacientes com conteúdo menor de plasmócitos em MM. Por outro lado, os doentes com MM que entraram em remissão após o tratamento apresentaram níveis iniciais mais baixos de CEPs do que os doentes com MM que não atingiram remissão. Ambos os níveis das células $CXCR4^+$ e PDGFR-β^+ foram correlacionados com os níveis de CEPs ao longo das fases de doença analisadas, sugerindo que estes recetores podem estar envolvidos em processos moleculares relacionados com as CEPs tais como recrutamento para localização do

tumor e proliferação em MM. Deste modo, os nossos resultados destacam pela primeira vez em amostras de MO arquivadas sequencialmente que as CEPs podem constituir um biomarcador para uma progressão agravada de GMSI para MM e uma pior resposta à terapia. Além disso, também destacamos a necessidade de estudar os mecanismos relacionados com a vasculogénese mediada por CEPs em MM.

Palavras-chave: células endoteliais progenitoras, gamopatia monoclonal de significado indeterminado, mieloma múltiplo, remissão, vasculogénese.

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Abbreviations

bFGF, basic fibroblast growth factor

BS, blocking solution

BSA, bovine serum albumin

BM, bone marrow

BMSC, bone marrow stromal cell

CD, cluster of differentiation

cEPC, circulating endothelial progenitor cell

cREM, complete remission

CSF1, colony-stimulating factor 1

CSF1R, colony-stimulating factor 1 receptor

CXCL12, C-X-C motif chemokine ligand 12

CXCR4, C-X-C motif chemokine receptor 4

DAPI, 4',6-diamidino-2-phenylindole

EC, endothelial cell

ECM, extracellular matrix

ELAM-1, endothelial-leukocyte adhesion molecule 1

EPC, endothelial progenitor cell

E-selectin, endothelial selectin

F, female

FLC, free light chain

Flk-1, fetal liver kinase 1

GS, goat serum

HSC, haematopoietic stem cell

ICAM, intercellular adhesion molecule

Ig, immunoglobulin

IGF, insulin-like growth factor

IL, interleukin

IPO, Instituto Português de Oncologia

ISS, international staging system

KDR, kinase insert domain receptor

LCA, leukocyte common antigen

LECAM-2, leukocyte-endothelial cell adhesion molecule 2

LFA-1, lymphocyte-function-associated antigen

M, male

Mc, monoclonal

MCAM, melanoma cell adhesion molecule

M-CSFR, macrophage colony-stimulating factor receptor

MGUS, monoclonal gammopathy of undetermined significance

MM, multiple myeloma

MMP, matrix metalloproteinase

MNC, mononuclear cell

M protein, monoclonal protein

MSC, mesenchymal stem cell

MVD, microvascular density

non-REM, not in remission

OB, osteoblast

OC, osteoclast

ON, overnight

PB, peripheral blood

PBS, phosphate buffered saline

PC, plasma cell

Pc, polyclonal

PDGF, platelet-derived growth factor

PDGFR-β, platelet-derived growth factor receptor β

PECAM-1, platelet endothelial cell adhesion molecule 1

pREM, partial remission

RANKL, receptor activator of nuclear factor-kappa B ligand

REM, remission

ROS, reactive oxygen species

RS, rabbit serum

RT, room temperature

SCFR, stem cell growth factor receptor

SDF-1α, stromal cell-derived factor 1α

SMM, smoldering multiple myeloma

TGF, transforming growth factor

TLR, toll-like receptor

treatMM, treated multiple myeloma

USA, united states of america

VCAM-1, vascular cell adhesion molecule 1

VE-cadherin, vascular endothelial cadherin

VEGF, vascular endothelial growth factor

VEGFR-2, vascular endothelial growth factor receptor 2

VLA-4, very-late-antigen 4

vWF, von Willebrand factor

%, percentage

?, inconclusive information

-, unavailable information

#, catalogue reference

1. Introduction

The bone marrow (BM) constitutes the primary site for haematopoiesis, a process through which blood cells are produced and the amount of each cell population that goes into circulation is regulated (Anthony and Link 2014). The BM is mainly composed by haematopoietic stem cells (HSCs) and non-haematopoietic stromal cells. While the HSC population originates all the blood cell types, the non-haematopoietic cells give rise to a variety of cells important to the BM maintenance (Wickramasinghe et al. 2011). The BM is characterized by a peculiar microenvironment that provides proper growth factors and conditions for the development of blood cells. In order to maintain an adequate haematopoiesis, the BM is dependent on a highly efficient blood supply (Iversen 1997). Several pathologies can affect the BM microenvironment, including multiple myeloma (MM), which is one of the most common hematologic malignancies. It is predominant in the elderly population and remains an incurable disease (Rajkumar 2014). While the first documented case of MM goes back to 1844, nowadays the annual incidence in the United States population is 4.3 per 100000 (Kyle and Rajkumar 2008). The disease is characterized by an increase in antibody producing plasma cells (PCs) in the BM, as well as overstimulation of blood vessel formation (Giuliani et al. 2011). In this disorder, endothelial progenitors may be recruited to the tumour surroundings, stimulating the vasculature development through vasculogenesis (Caiado and Dias 2012). Since studies about MM vasculogenesis are lacking, we focused on the importance of the alterations in the BM microenvironment and the formation of blood vessels while taking a special interest on endothelial progenitors.

1.1. The bone marrow and its microenvironment

The BM is found amongst bone trabeculae of spongious bone and in the diaphysis of long bones. This bone compartment constitutes the main site of haematopoiesis, in which all blood cells are originated from a common pluripotent stem cell. Haematopoiesis can be divided in initial cell proliferation, commitment to a cell lineage, and cell differentiation, where biochemical, functional, and structural changes give rise to a specific cell type (Wickramasinghe et al. 2011). The BM microenvironment, or stroma, is mainly composed by both HSCs and non-haematopoietic or stromal cells. HSCs have unlimited self-renewal and are able to differentiate in all the blood cell types from the myeloid and lymphoid lineages (Krause 2002). The rich BM microenvironment is composed by several cell populations. Facing the bone matrix, there are endosteal osteoblasts (OBs) and osteoclasts (OCs) that are responsible for bone formation and resorption, respectively. The substantial blood vessels are lined by endothelial cells

(ECs). Adipocytes constitute the cells with the largest dimensions and their amount is inversely related with the BM cellularity. The population of BM stromal cells (BMSCs) englobes OBs, ECs, mesenchymal stem cells (MSCs), among others. MSCs are non-haematopoietic stromal cells that only constitute about <0.01% of the BM. These stromal cells can differentiate into multilineages, being able to originate OBs, chondrocytes and adipocytes (Wickramasinghe et al. 2011). The use of the definition of BMSCs and MSCs amongst the literature is not consensual, as some authors choose to refer to both as the same cell population, even though they constitute different cell types (Nemeth and Mezey 2015). All mentioned cells are connected through a network of extracellular matrix (ECM) (Klamer and Voermans 2014, Romano et al. 2014), containing soluble factors like cytokines and where important cell-cell interactions and cell-ECM interactions take place (Krause 2002), as illustrated in Figure 1. Inside the BM, distinct microenvironments can be found. These microenvironments are called niches and their function is to regulate the outcome of the cells they harbour (Balderman and Calvi 2014). These niches constitute the endosteal/osteoblastic niche and the vascular/arteriolar niche (Guerrouahen et al. 2011). The endosteal niche is located in the inner part of the bone cavity, near the endosteum, the layer that internally lines the bone (Bydlowski et al. 2013). To confirm the behaviour of HSCs in the BM, Xie *et al*. transplanted HSCs and observed that HSCs home to the BM endosteum, through its vasculature. There, HSCs are maintained, but can also undergo proliferation upon BM damage (Xie et al. 2009). As endosteum OBs are the main component of the niche, they contribute to HSCs quiescent state by down-regulating HSCs proliferation and differentiation (Nilsson et al. 2005) and by inducing bone adhesion, through angiopoietin-1 secretion (Arai et al. 2004). Moreover, MSCs act as a supportive niche for HSCs by participating in their homing and maintenance (Battiwalla and Hematti 2009, Mendez-Ferrer et al. 2010). Interestingly, HSCs that are more mature and have a higher proliferation level disperse from the endosteal niche (Lo Celso et al. 2009). During stress situations, OCs cleave C-X-C motif chemokine ligand (CXCL)12/stromal cell-derived factor 1, which stimulates HSCs mobilization (Kollet et al. 2006). Then, HSCs migrate from the endosteum and reach a vascular region where they start proliferation. This region constitutes the vascular/arteriolar niche, in which ECs are a strong component (Guerrouahen et al. 2011). It has been considered that this niche is divided in quiescent arteriolar niche and cycling sinusoidal niche (Klamer and Voermans 2014). The BM has a unique blood supply that differs from the remaining circulatory system. The main blood source constitutes the nutrient artery, which enters through the cortex (bone periphery) leading to the sinusoids. These are highly permeable capillaries formed by ECs with a discontinuous basement membrane (Iversen 1997, Marenzana and Arnett 2013).

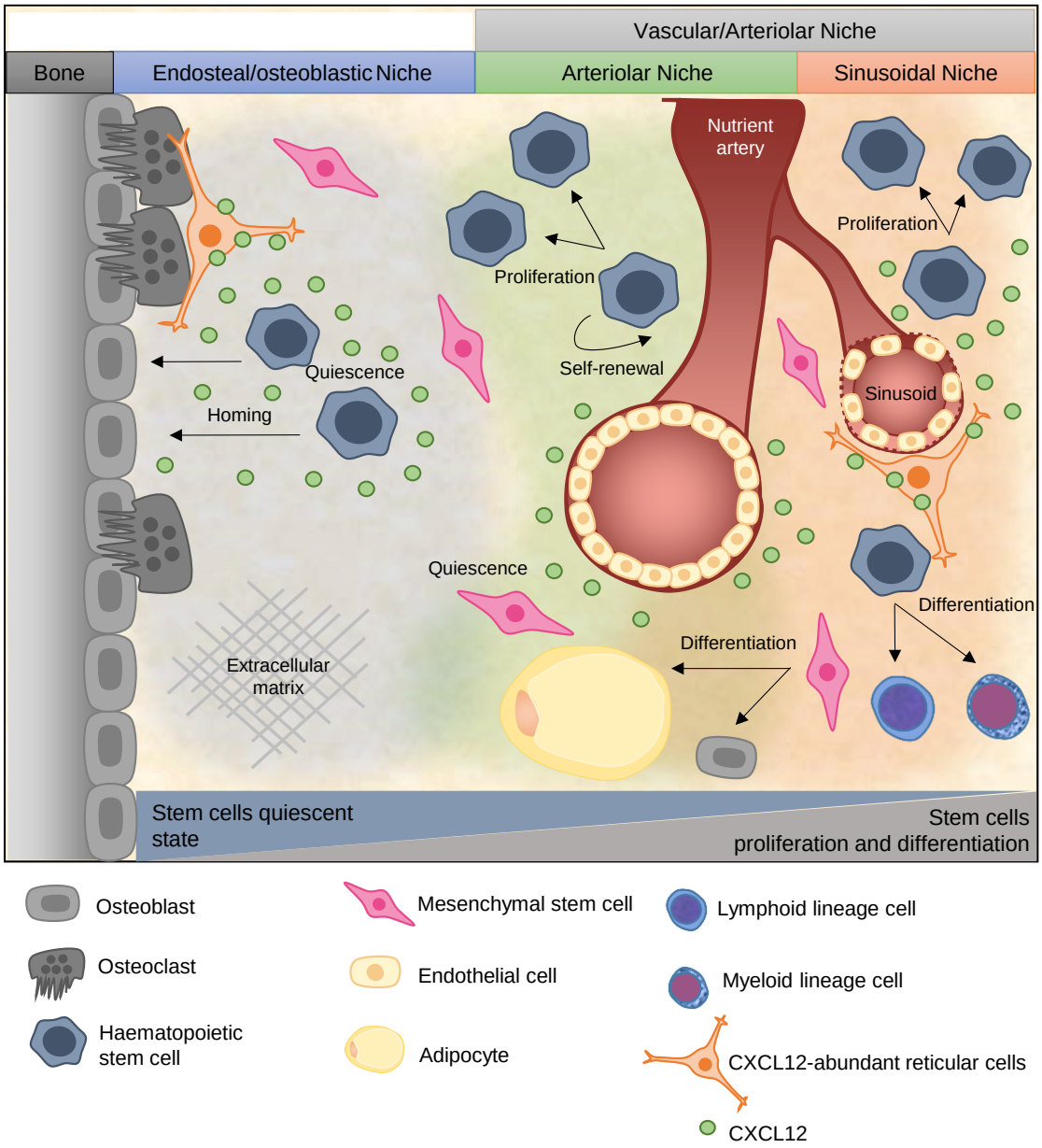

Figure 1: Schematic representation of the bone marrow (BM) microenvironment and respective niches. Close to the endosteum, the endosteal or osteoblastic niche is characterized by the presence of bone osteoblasts and osteoclasts. Osteoblasts induce haematopoietic stem cells homing into this location and contribute to their quiescent state. The location of stem cells is secured through C-X-C motif chemokine ligand (CXCL)12 and CXCL12-abundant reticular cells, near both the endosteum and the vasculature. The BM vasculature plays a key role in the proliferation and differentiation of stem cells. The source of the BM vasculature is named the nutrient artery which gives origin to sinusoids, capillaries that lack a continuous endothelial lining. The arteriolar niche has haematopoietic stem cells in an intermediate self-renewing state, while the sinusoidal niche is thought to contribute more significantly to both haematopoietic and mesenchymal stem cells proliferation and differentiation into myeloid and lymphoid cells, and osteoblasts and adipocytes, respectively.

The arteriolar niche is thought to be closer to the endosteum, where it contributes to the quiescent state of HSCs, while the sinusoidal niche may be spread through the BM and contributes more significantly to haematopoietic stem and progenitor cells proliferation and differentiation (Guerrouahen et al. 2011, Klamer and Voermans 2014). The arterial blood vessels microenvironment has a characteristic low reactive oxygen species (ROS) level, while the highly permeable sinusoids have higher ROS levels, which promote HSCs to migrate towards the higher ROS state and differentiate (Itkin et al. 2016). In the arteriolar quiescent niche, MSCs also remain in their quiescent state (Kunisaki et al. 2013). In addition, the proximity of HSCs to the vasculature is maintained mainly by the C-X-C motif chemokine ligand (CXCL)12 and C-X-C motif chemokine receptor (CXCR)4 interaction, as CXCL12-abundant reticular cells are found near sinusoidal ECs or close to the endosteum. CXCL12 is thus essential to HSCs niche-homing (Sugiyama et al. 2006).

1.2. Formation of blood vessels

Blood vessels are constituted by a monolayer of ECs that form a vascular wall, which is surrounded by the basement membrane. Embedded in this membrane, are mural cells such as smooth muscle cells in larger vessels and pericytes at pre-capillary arterioles, capillaries, and post-capillary venules (Sá-Pereira et al. 2012). Additionally, the ECM provides a structural framework to the blood vessels (Jacob et al. 2001). In the adult, the vasculature remains mostly in a quiescent state (Charpentier and Conlon 2014). The formation of new blood vessels in the adult constitutes the process of neovascularization. Blood vessels can be formed either by vasculogenesis or angiogenesis. Although vasculogenesis is characteristic of embryonic development, postnatal vasculogenesis has also been demonstrated, in both physiological and pathological conditions (Asahara et al. 1999, Morales-Ruiz and Jiménez 2005). During embryonic development, vasculogenesis is defined as *de novo* differentiation of mesodermal precursors into precursor ECs, also known as angioblasts or endothelial progenitor cells (EPCs), which will ultimately differentiate into ECs. EPCs proliferate and integrate into the primary capillary plexus, a primate vascular network. This initial network is then completed through angiogenesis (Asahara et al. 1999, Papetti and Herman 2002) that represents the most common process of vascular development. In the adult, physiological angiogenesis is mostly related to the ovarian cycle and wound healing. On the other hand, in pathological events, the absence of angiogenesis is characteristic of cardiac failure, while excessive angiogenesis is found in chronic inflammation and cancer (Griffioen 2012). This process of blood vessel development is present in tumour growth, invasion and metastasis, and is also considered to have a key role in several

haematological malignancies (Otjacques et al. 2011). Angiogenesis can occur by sprouting angiogenesis or intussusceptive angiogenesis. Sprouting angiogenesis begins with the degradation of the ECM and basement membrane by proteolytic enzymes. ECs migrate, adhere, and proliferate in order to form a vascular sprout that is stabilized by a basement membrane and the recruitment of mural cells. Intussusceptive angiogenesis is characterized by the division of pre-existing vessels through transcapillary tissue pillars that are stabilized by the invagination of the ECM and pericytes (Morales-Ruiz and Jiménez 2005). The angiogenic process is highly regulated through soluble factors that are mainly responsible for controlling ECs proliferation, migration and endothelium stability, as well as by membrane-bound factors that control cell adhesion and by chemokines that are involved in chemotaxis (Papetti and Herman 2002, Jakob et al. 2006).

1.3. Endothelial progenitor cells

The term EPCs was first reported in 1997 by Asahara *et al*. (Asahara et al. 1997). Ever since, EPCs are defined as BM derived progenitors with high proliferative ability that have the potential to differentiate into cells of the endothelial lineage (Laurenzana et al. 2015). It was estimated that EPCs represent up to 26% of ECs in recently formed vessels (Murayama et al. 2002). BM-derived EPCs participate in postnatal vasculogenesis, in physiological processes such as tissue growth, and in pathological events like myocardial ischemia, stroke, artherosclerosis and cancer (Caiado and Dias 2012, Moschetta et al. 2014), a topic that will be developed later in the review. Furthermore, these cells contribute to re-endothelialisation in scenarios of tissue injury, which is why EPCs have been considered as an important tool for therapy in tissue repair (Tenreiro et al. 2016). EPCs also aid in maintaining the vasculature through the production of angiogenic factors that stimulate the proliferation, function and survival of ECs. Therefore, they have an indirect but important role in angiogenesis (Laurenzana et al. 2015). It is important to highlight that the notion of angiogenesis is often used when referring to any new blood vessel formation (Kovacic et al. 2008). Thus, EPCs are repeatedly connected with that process even though EPCs are defined as key players in vasculogenesis and not angiogenesis, as stated in the previous section. EPCs are considered to develop from hemangioblasts, which also give origin to HSCs (Moschetta et al. 2014). EPCs are kept in a quiescent BM niche that is thought to have low oxygen tension and an elevated amount of CXCL12 that is responsible for maintaining them (Balaji et al. 2013). In cases of trauma or wound-healing generating hypoxia, EPCs are stimulated to leave this niche, reach the proliferative niche and then are able to go into circulation (Velazquez 2007). Then, EPCs home into their target tissue and are activated.

Afterwards, EPCs adhere to the ECs of the vessel and begin transendothelial migration for vascular remodelling. After crossing the endothelial monolayer, EPCs migrate through the basement membrane and ECM, a mechanism that depends on extracellular proteases. When reaching the vessel remodelling site, EPCs differentiate into ECs and/or interact with the ECs. Although the functional activity of EPCs is mostly under investigation, it is considered that their differentiation involves adhesion to the ECM components controlled by integrins, proliferation and survival induced by growth factors, and maturation and acquisition of the endothelial phenotype (Caiado and Dias 2012). The peculiar properties of these cells in the development of blood vessels have attracted special interest and, although there are many studies about them, the knowledge about these cells is far from being complete. In addition, the precise process of origin of EPCs remains to be fully clarified, mainly due to the controversial identification of these cells, as it is portrayed in the next section.

1.3.1. Characteristic markers

Until today, EPCs remain with no uniform definition and no specific cell-surface antigen (Yoder 2012, Tenreiro et al. 2016). For that reason, it is important to underline the developments in this topic. After isolation from peripheral blood (PB), EPCs were firstly identified with the markers cluster of differentiation (CD)34 and vascular endothelial growth factor receptor (VEGFR)-2 (Asahara et al. 1997). Since then, besides being distinguished based on the expression of markers, EPCs are also isolated based on their functional and clonal expansion features (Balaji et al. 2013). EPCs display the capacity to phagocyte low density lipoprotein LDL, and bind to *Ulex europaeus* lectin-1 (Song et al. 2010). In order to characterize EPCs, researchers usually isolate PB, BM or cord blood mononuclear cells (MNCs), and perform the respective culture in specific cell growth medium in order to outgrow putative EPCs or, alternatively isolate and identify putative EPCs through multiple markers expression (Eggermann et al. 2003, Braunstein et al. 2006, Song et al. 2010, Yang et al. 2011, Amini et al. 2012). Several authors have used and attributed different markers for putative EPCs, as depicted in Table 1.

Table 1: Markers expressed by putative endothelial progenitor cells and/or whose expression has been deemed characteristic of putative endothelial progenitor cells

Marker	Other names	Cellular expression	Function	Reference
CD34	-	Transmembrane	Adhesion molecule between EC and haematopoietic stem cells Cell migration	(Ria et al. 2008, Shi and VandeBerg 2015)
VEGFR-2	Flk-1 KDR CD309	Transmembrane	Receptor 2 for VEGF. Involved in the formation of blood vessels	(Eggermann et al. 2003, Case et al. 2007, Timmermans et al. 2007, Fadini et al. 2008)
CD133	Prominin-1 AC133	Transmembrane	Unclear function	(Peichev et al. 2000, Shmelkov et al. 2005, Ria et al. 2008)
CD45	LCA	Transmembrane	T and B cell protein tyrosine phosphatase involved in lymphocyte activation and proliferation	(Trowbridge and Thomas 1994, Yoder 2012)
CD31	PECAM-1	Transmembrane and soluble	Adhesion molecule in EC intercellular junctions and angiogenesis	(Peichev et al. 2000, Kalinowska and Losy 2006, Song et al. 2010, Amini et al. 2012)
CD146	MCAM	Transmembrane and soluble	Adhesion molecule in EC adherens junctions	(Peichev et al. 2000, Bardin et al. 2001)
E-selectin	CD62E ELAM-1 LECAM-2	Transmembrane and soluble	Adhesion molecule in EC	(Peichev et al. 2000, Ley 2003)
VE-cadherin	Cadherin 5 type 2 CD144	Transmembrane and soluble	Adhesion molecule in EC	(Peichev et al. 2000, Eggermann et al. 2003, Ria et al. 2008, Vestweber 2008)
Tie-2	Tyrosine-protein kinase receptor	Transmembrane	Receptor tyrosine kinase, involved in proliferation and differentiation	(Schnürch and Risau 1993, Fadini et al. 2008, Ria et al. 2008)
c-kit	SCFR Tyrosine-protein kinase Kit CD117	Transmembrane	Cytokine receptor involved in cell survival, proliferation and differentiation	(Nocka et al. 1990, Peichev et al. 2000, Cananzi and De Coppi 2012)
CD14	-	Transmembrane and soluble	Co-receptor for the TLR, Innate immune response	(Eggermann et al. 2003, Lau et al. 2014)
CD115	CSF1R M-CSFR	Membrane	Receptor for the CSF1	(Fadini et al. 2008)

CD105	Endoglin	Membrane	TGF-β receptor complex receptor	(Cheifetz et al. 1992, Eggermann et al. 2003)
vWF	-	Soluble	Platelet adhesion molecule	(Eggermann et al. 2003, Lenting and Christophe 2015)
CXCR4	CD184	Transmembrane	Chemokine receptor for SDF-1α/CXCL12	(Peichev et al. 2000, Kucia et al. 2004)

CD, cluster of differentiation; CSF1, colony-stimulating factor 1; CSF1R, colony-stimulating factor 1 receptor; CXCL12, C-X-C motif chemokine ligand 12; CXCR4, C-X-C motif chemokine receptor type 4; EC, endothelial cell; ELAM-1, endothelial-leukocyte adhesion molecule 1; E-selectin, endothelial selectin; Flk-1, fetal liver kinase 1; KDR, kinase insert domain receptor; LCA, leukocyte common antigen; LECAM-2, leukocyte-endothelial cell adhesion molecule 2; M-CSFR, macrophage colony-stimulating factor receptor; MCAM, melanoma cell adhesion molecule; PECAM-1, platelet endothelial cell adhesion molecule 1; SCFR, stem cell growth factor receptor; SDF-1α, stromal cell-derived factor 1α; TLR, toll-like receptor; TGF, transforming growth factor; VE-cadherin, vascular endothelial cadherin; VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor 2; vWF, von Willebrand factor.

Several cell populations of putative EPCs have been described, which makes it difficult to establish connections between the existing studies. Most studies have focused on isolating putative EPCs from PB and detailed studies exploring EPCs in the BM are rare to find. The types of putative EPCs have been mostly defined based on the maturation state and/or according to cells isolated from MNCs *in vitro* (Fig. 2)*,* while differentiation *in vivo* remains elusive. Indeed, it remains a challenge to establish equivalencies between EPCs *in vivo* and *in vitro,* since EPCs in culture may gain or lose characteristics that are not present in non-cultured cells. EPCs can be classified based on maturation state, proliferative potential, endothelial markers, morphology and capacity to form blood vessels (Moschetta et al. 2014). Peichev and colleagues observed that $CD34^+VEGFR\text{-}2^+$ cells also express CD133 and have the capacity to migrate and differentiate into adherent mature ECs. As these cells become mature ECs, they lose CD133 expression (Peichev et al. 2000). Supporting this, Quirici *et al*. isolated EPCs from BM $CD133^+$ cells that later gave rise to ECs. After 3 weeks of culture, these cells were negative for CD45 and CD14 and positive for several EC markers such as *Ulex europaeus* lectin-1, von Willebrand factor (vWF), CD105, endothelial selectin, vascular cell adhesion molecule 1 and vascular endothelial (VE)-cadherin (Quirici et al. 2001). On the other hand, $CD34^+$ cells were isolated from MNCs from human umbilical cord blood and it was reported that $CD34^+CD45^+$ cells formed haematopoietic progenitor cells, while $CD34^+CD45^-$ formed EPCs with endothelial colony forming activity. Thus, the authors of this study stated that true EPCs had no expression of the haematopoietic lineage-specific and common leukocyte antigen CD45 (Case et al. 2007). Contrarily to the previously

mentioned study, functional EPCs were attained from $CD34^+$ cells isolated from mouse BM MNCs and these EPCs were positive for CD45 after culture (Yang et al. 2011). Hence, these contradictory reports make for a difficult interpretation, more specifically, of the relevance of the expression of CD45, which is characteristic of haematopoietic cells (Amini et al. 2012). Even so, the combination of CD34, CD133 and VEGFR-2 for the identification of EPCs is generally used (Massa et al. 2005, Caiado et al. 2011, Blix et al. 2015).

Ultimately, when identifying EPCs, it is crucial to bear in mind the methodology used for their isolation and identification, to not intensify the incoherency regarding the terminology and the protocols for isolating putative EPCs. The markers used in the several studies to identify EPCs are not exclusive to EPCs as they can also be expressed by HSCs and, therefore, do not allow a clear and effective identification of these cells (Fadini et al. 2008). This field of investigation deserves further research considering the potential and importance that these cells harbour, the interest in a better knowledge of their characterization, as well as in the development of new techniques for their isolation and differentiation in EPCs with consistent and reliable phenotypes.

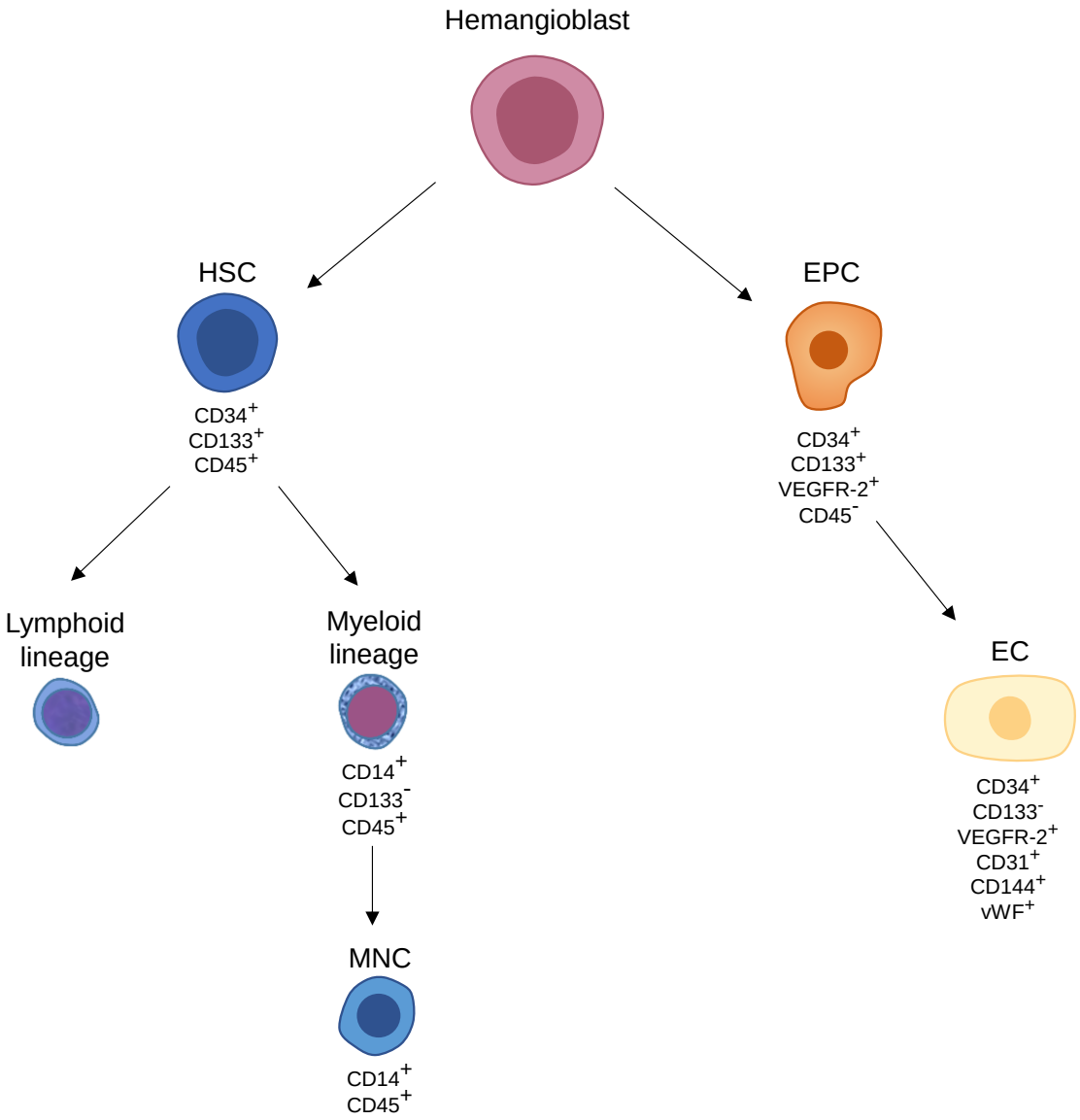

Figure 2: Schematic representation of the origin and differentiation of endothelial progenitor cells (EPCs) and haematopoietic stem cells (HSCs) populations and their respective characteristic markers. Hemangioblasts can differentiate into HSCs and EPCs. HSCs give rise to cells of the lymphoid and myeloid lineages. In *in vitro* culture, mononuclear cells (MNCs) can be isolated and differentiated into putative EPCs. True EPCs originated from the hemangioblast express cluster of differentiation (CD)34, vascular endothelial growth factor receptor (VEGFR)-2 and CD133. Ultimately, EPCs give rise to endothelial cells (ECs), which express typical EC markers such as CD34, VEGFR-2, CD31, CD144 and von Willebrand factor (vWF).

1.4. Multiple myeloma

MM represents about 10% of all hematologic malignancies, for which there is still no cure. Patients are usually diagnosed at 65 years old and have a median survival of 3 to 4 years (Rajkumar 2014). MM is characterized by BM accumulation of malignant PCs and high levels of a monoclonal immunoglobulin (Ig), mostly IgG or IgA, or free light chains (FLC) that are detected in blood and/or urine (Bianchi and Anderson 2014). PCs

constitute terminally differentiated lymphoid B cells, which are located in the BM and the medulla of lymph nodes. These cells synthesize Igs that act as antibodies against antigens. When clonal expansion of PCs is altered, it leads to an increased synthesis of a monoclonal Ig that translates as an elevated monoclonal peak in serum electrophoresis. The visual anomalies in PCs include abnormal chromatin network, irregular nuclear outline and cytoplasm colour, and inclusions with different origins connected to irregular trafficking or catabolism related to Ig synthesis by PCs. The diverse morphological characteristics constitute various types of PCs. For instance, immature PCs are named plasmablasts, which are characteristic of patients with higher BM PCs levels and proliferation rate. On the other hand, patients with predominantly mature PCs are usually correlated with a better prognostic. Multiple nuclei can also be observed in PCs, as they are a part of PC burden (Ribourtout and Zandecki 2015). Most importantly, MM is associated with lytic bone disease, due to osteolytic resorption and suppression of bone formation. This leads to the main cause of malaise in MM, namely the excruciating bone pain (Heider et al. 2006).

Clinical staging of malignancies such as MM allows a reliable classification of the disease progression and prediction of patient survival. As a matter of fact, MM belongs to a spectrum of distinct PC disorders, as illustrated in Figure 3. The first manifestation of this disease consists of monoclonal gammopathy of undetermined significance (MGUS), followed by the intermediate smoldering MM (SMM) and, lastly, MM. These stages can be clinically distinguished through the percentage (%) of BM PCs, serum monoclonal protein (M protein) and organ end damage (Kyle 1978, Kyle and Greipp 1980, Kyle et al. 2002, Bianchi and Anderson 2014).

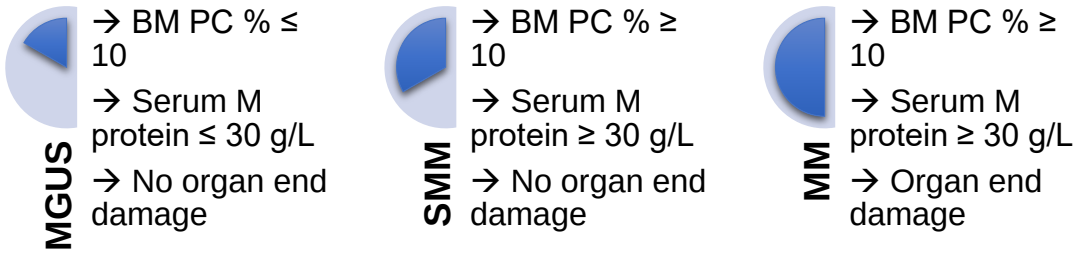

Figure 3: Stages of multiple myeloma progression and respective diagnosis parameters. BM PCs %, percentage of plasma cells in the bone marrow; MGUS, monoclonal gammopathy of undetermined significance; M protein, monoclonal protein; MM, multiple myeloma; SMM, smoldering multiple myeloma.

MGUS is characterized by serum monoclonal Ig of 30 g/L or less and 10% or less of BM PCs, without lytic bone lesions, and no evidence of anaemia, hypercalcaemia and renal insufficiency. It is thought that factors such as genetic changes, several cytokines important in myeloma bone disease and angiogenesis in the BM are responsible for the progression of MGUS to MM, which risk is around 1% per year (Kyle and Rajkumar 2006). MGUS diagnosis is also made through the amount of M protein in serum protein electrophoresis, by immunofixation or an irregularity in a serum FLC assay (Kyle et al. 2004). However, this disorder ends up underdiagnosed, due to the absence of MM symptoms. The asymptomatic stage between MGUS and MM consists of SMM. It was firstly identified in patients with a % of PCs and levels of M protein higher than in MGUS (BM PCs ≥ 10%; M protein level ≥ 30 g/L) that presented no symptoms and remained clinically stable for 5 or more years (Kyle and Greipp 1980). The progression from this state to MM can vary from 2 to 19 years, depending on several factors such as serum M protein levels, presence of IgA isotype, serum FLC ratio, % of BM and PB PCs, cytogenic abnormalities and Bence Jones proteinuria (Gao et al. 2015, Gentile et al. 2015). After the MM malignancy is set up, the symptoms usually include fatigue, bone pain, anaemia, renal failure, hypercalcemia and even peripheral neuropathy and amyloid light chain amyloidosis. The diagnosis is based on BM PCs ≥ 10%, serum M protein ≥ 30 g/L and the presence of end-organ failure (Bianchi and Anderson 2014). MM can be classified according to two staging systems: the Durie-Salmon and the International Staging System (ISS). Since the Durie-Salmon system quantifies the tumour burden but not the bone lesions, the ISS fills that gap by dividing patients into fractions based upon the prognostic factors β2-microglobulin and serum albumin levels (Durie and Salmon 1975, Greipp et al. 2005).

MM is known for its clinical and biological heterogeneity, which is responsible for inconstant response to treatment. Biomarkers can be used to distinguish this heterogeneity and to choose the most adequate treatment option. Biomarkers are defined as a biochemical, cellular/molecular substance or characteristic that is correlated to a regular or pathogenic biological process, or to a response to a certain therapy, and can be measured in an accurate and reproducible way (Naylor 2005). The most commonly used biomarkers for MM diagnosis are PC labelling index, BM-infiltration rate, serum M protein and FCL, and levels of albumin and β2-microglobulin. More uncommonly, are calcium and serum creatinine levels, and cytogenetic abnormalities (Kastritis et al. 2013, Rajshenkhar and Shaji 2015). Even with the high amount of biomarkers for MM, there is a need to complete this list with more sensitive biomarkers in order to deal with the progression from an asymptomatic state to MM, which is still

unpredictable due to the characteristic heterogeneity of MM (Landgren and Morgan 2014). Moreover, with increasing MM knowledge and therapy options, new biomarkers can help physicians to identify which patients would benefit from a specific therapeutic option and the response the patient may display.

There is still no therapy for asymptomatic MM or SMM and so it is advisable a close follow-up until MM symptoms evolve (Gentile et al. 2015). Regarding MM, in 1983 the median survival of MM was less than 2 years with only 2.2% of the patients surviving for longer than 10 years with chemotherapy (Kyle 1983). Over the last decade, new therapies have emerged to treat MM, which resulted in a better outcome (Chng et al. 2014). The therapies currently available to attenuate MM symptoms consist of alkylating agents, immunomodulatory drugs such as thalidomide, proteasome inhibitors like bortezomib, immunotherapy with monoclonal antibodies and vaccines, chemotherapy, long-acting steroids and, in the worst cases, autologous stem cell transplant (Bianchi and Anderson 2014, Rajkumar 2014). Nowadays, it is considered that standard risk patients have an overall survival of 6-7 years, whereas high risk patients only seem to survive for 2-3 years even with autologous stem cell transplantation (Rajkumar 2014). Even with the large amount of therapies available, patients do not maintain remission (REM) for long and relapse is expected (Kurtin 2013). Hence, despite the latest evolution in MM treatment, it remains an incurable disease, which demands new therapeutics not only concerning the tumour cells, but also to their surrounding microenvironment.

1.4.1. Compromised bone marrow microenvironment

Since MM PCs accumulate in the BM, its microenvironment plays an important role in MM progression. In fact, BMSCs and MSCs support the development of MM, whether it is by cell-cell interactions or by cytokines secretion. MM MSCs and their progenitors differ from normal MSCs, as they greatly support the growth and survival of MM PCs (Wallace et al. 2001, Corre et al. 2007). MM PCs show higher levels of proliferation when incubated with MM MSCs, namely through expression of translation initiation factors, which contributes highly to the transformation of normal PCs to malignant PCs (Attar-Schneider et al. 2015). MM PCs and MSCs communicate mainly through the CXCR4 signalling pathway (Feng et al. 2010). Not only do BMSCs support the growth of tumour cells, but they also contribute to the MM bone disease, as described below. This hallmark of MM consists in the imbalance between bone formation and degradation. It is connected to chronic pain, bone fractures, spinal cord compressing and, thus, a poor life quality (Walker et al. 2014). Myeloma bone disease is achieved through the inhibition of OBs, which are responsible for bone formation, and the

promotion of OCs that are involved in bone resorption. There is an increase in OBs inhibitors from the Wnt pathway, which is essential for OB differentiation, therefore reducing OBs activity. Moreover, the dysregulation of interleukin (IL)-3 and IL-7 expression leads to a disturbance of OBs survival. On the other hand, the OCs regulators tumour necrosis factor-related apoptosis-inducing ligand and receptor activator of nuclear factor-kappa B ligand (RANKL) levels are higher in MM patients (Kristensen et al. 2014, Romano et al. 2014). RANKL is secreted when MM PCs adhere to BMSCs and bind to its receptor RANK in OCs progenitor cells, leading to stimulation of OCs activity, differentiation and bone resorbing. BMSCs also secrete osteoprogesterin, which inhibits OCs differentiation by RANK binding. However, in MM, osteoprogesterin is present in low levels, which leads to a further promotion of OCs differentiation. IL-6 also promotes the action of osteoclastogenic factors and bone degradation (Romano et al. 2014, Walker et al. 2014). Interestingly, Lawson *et al*. recently reported that, even though most of MM PCs remained circulating in the BM, colonizing MM PCs migrated outwards endocortical bone and remained in the endosteal bone niche. Furthermore, the endosteal niche inhibits MM PCs growth as OBs contribute to suppress MM PCs proliferation and to keep MM PCs in a dormant state. Remarkably, this inhibition is reversible, as OCs activity and bone resorption lead to a reduction in bone surface and release of MM PCs from the endosteal niche, further contributing to tumour development (Lawson et al. 2015).

1.4.2. Malignant angiogenesis

Both angiogenesis and vasculogenesis have been subject of discussion in MM (Giuliani et al. 2011). Tumour angiogenesis differs greatly from physiologic angiogenesis as it is characterized by an increase in ECs proliferation and in the levels of the factors that regulate this process. In tumours, angiogenesis results in an abnormal and aberrant vasculature with heterogeneous ECs, irregular blood flow, instability and elevated permeability, as is the case of MM (Jakob et al. 2006, Giuliani et al. 2011). Early on and until nowadays, it is considered that an “angiogenic switch” occurs from MGUS to MM. In fact, MGUS is considered an avascular phase, whereas the development of blood vessels is characteristic of MM (Vacca et al. 1999, Calcinotto et al. 2015). This switch is caused by the malignant cells and leads to the detachment of pericytes, vessel dilation and sprouting (Giuliani et al. 2011). Interestingly, MGUS BM samples showed the capacity to decrease angiogenesis, while samples from SMM and MM patients stimulated angiogenesis *in vitro* (Kumar et al. 2004). These results point to a switch in the BM involving pro- and anti-angiogenic factors that participate in paracrine interactions between MM PCs, ECs and BMSCs (Jakob et al. 2006). In fact, assessment of microvascular density (MVD) and expression of angiogenic factors demonstrated that

BM angiogenesis is increased in MM (Di Raimondo et al. 2000, Lee et al. 2015). In 1994, it was firstly demonstrated that BM MVD was significantly increased as well as BM angiogenesis in patients with MM in comparison with MGUS (Vacca et al. 1994). Numerous studies have since suggested the prognostic potential of BM MVD, as higher levels correlate with MGUS evolution to MM and shorter progression-free survival. BM MVD was also connected with the level of BM PCs and PC labelling index, as reviewed by Giuliani *et al.* (Giuliani et al. 2011).

1.4.3. Endothelial progenitor cells-induced vasculogenesis

EPCs have a central role in vasculogenesis, as previously mentioned. These cells have been studied in several haematological malignancies as non-Hodgkin's lymphoma, leukaemia and MM. Since EPCs have the same mesodermal progenitor as HSCs, which will originate the malignant cells in MM, it was proposed that the origin of EPCs may be from the same malignant clone as haematopoietic cancer cells (Moschetta et al. 2014). The analysis of neovascularization through MVD requires a BM biopsy, which is a very invasive procedure. On the other hand, EPCs can be isolated and evaluated less invasively from PB, so research turned to exploit that method (Zhang et al. 2005). Accordingly, EPCs in the context of MM were firstly mentioned by Zhang *et al*., in 2005, regarding circulating EPCs (cEPCs) in PB. The study showed that cEPCs levels were higher in MM in comparison to healthy controls and that these levels were correlated with those of M protein and β2-microglobulin, suggesting that EPCs can constitute a biomarker for MM progression (Zhang et al. 2005). Later on, it was confirmed that cEPCs levels in MM were correlated with the evolution of the disease from ISS I to ISS III stage (Bhaskar et al. 2012). A recent study evaluated PB and BM levels of EPCs in healthy individuals and in SMM and MM patients. The control levels were significantly lower in comparison to the disease levels in the two compartments. In PB, the EPCs levels in SMM were slightly lower than in MM, while in the BM, SMM levels were slightly higher than in MM. This study shows that even in early stages of MM, the mobilization and proliferation of EPCs in the BM is substantial in comparison to healthy conditions (Moschetta et al. 2016). BM cells positive for VEGFR-2, a marker used for EPC identification, were also depicted to be higher in MM patients (with Salmon-Durie stage II/III, active disease and BM-infiltration rates of ≥20%) when comparing to MGUS patients (Udi et al. 2011). Regarding treatment, only BM $CD34^{+}VEGFR\text{-}2^{+}$ EPCs levels were significantly higher in MM in comparison to REM (Udi et al. 2011). Thalidomide treatment led to a decline in cEPCs (Zhang et al. 2005). With chemotherapy**,** cEPCs declined in responders to therapy, while they increased in non-responders (Bhaskar et al. 2012). More recently, concerning the combination of bortezomib with dexamethasone, higher

levels of cEPCs were correlated with a later response to this treatment (Wang et al. 2015), which underlines cEPCs potential as a prognostic biomarker. Additionally, in autologous stem cell transplant, higher EPCs levels in PB stem cell autografts were associated with a lower overall survival after the therapy (Blix et al. 2015). Regarding different treatments and their effect on EPCs, the levels of BM EPCs were higher when novel agents were administrated (thalidomine, lenalidomine and bortezomib) in comparison to stem-cell transplant treatment and to stem cell transplant plus novel agents (Udi et al. 2011). There seems to be lack of significant information about the amount of EPCs in the BM regarding different stages of MM, as most studies have focused on PB EPCs. Furthermore, since different responses to therapy are associated with different levels of EPCs, these levels could be used in order to predict the patients response to treatment and which treatment option would be best in a particular scenario. Still, this topic remains much unexplored, as there is an absence of studies regarding BM levels of EPCs and their correlation to the different therapies available.

Mobilization path in the bone marrow

The exact mechanisms related to the path EPCs make in the BM in the malignant scenario of MM are still to be studied. Considering that the tumour site is located within the BM, it is uncertain if EPCs need to intravasate into the vasculature to reach the tumour location, as they do to reach a location distant from the BM, or if EPCs simply migrate to the tumour location within the BM itself, as illustrated in Figure 4A. Even so, several critical phases are involved in vasculogenesis. First, EPCs must leave their BM niche and be recruited to the tumour microenvironment (Fig. 4B-C). Upon reaching the vasculature surrounding the tumour niche, EPCs home to the vasculature by ECs stimulation (Fig. 4D). EPCs adhere to ECs and initiate transendothelial migration (Fig. 4E). Upon integrating the endothelium, EPCs differentiate into mature ECs (Fig. 4F). This process is further completed with the stabilization of blood vessels provided by pericytes (Fig. 4G).

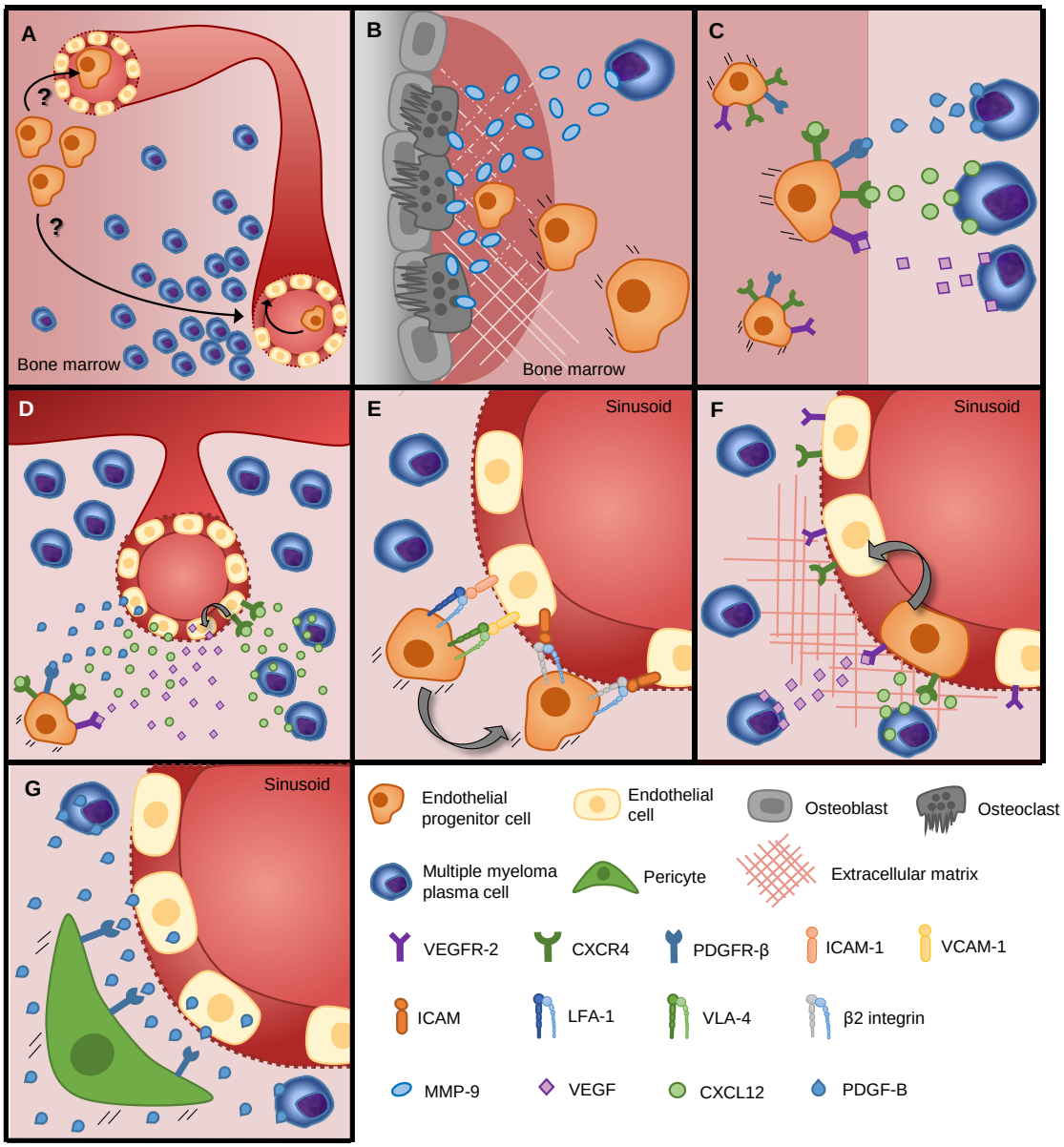

Figure 4: Schematic representation of the proposed mechanisms for endothelial progenitor cells (EPCs) mobilization in the bone marrow (BM) and neovascularization in multiple myeloma (MM). EPCs are formed in the BM, where MM plasma cells (PCs) proliferate. EPCs are mobilized to the tumour location, but the question to if EPCs travel through the blood stream or the BM itself remains unanswered (A). Within these hypotheses, initially, EPCs remain in the quiescent niche near the endosteum, osteoblasts and osteoclasts. EPCs are released from the quiescent niche mainly due to matrix metalloproteinase (MMP)-9 secreted by both osteoclasts and MM PCs, which degrade the extracellular matrix in the niche, facilitating EPCs mobilization (B). EPCs are mobilized to the tumour site attracted by the ligands C-X-C motif chemokine ligand (CXCL)12, vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF)-B secreted by MM PCs that bind to the respective receptors C-X-C motif chemokine receptor (CXCR)4, vascular endothelial growth factor receptor (VEGFR)-2 and PDGF receptor (PDGFR)-β expressed by EPCs (C). Once at the vasculature site, endothelial cells (ECs) induce EPCs homing to the vasculature mediated by the same molecules mentioned in (B). Both CXCL12 and PDGF-B can be produced by ECs, whereas the production of VEGF by these cells can stimulated by CXCL12 secreted by MM PCs (D). Then, EPCs must adhere to the endothelial wall of the vessel being

remodelled. This process can be done through integrins lymphocyte-function-associated antigen (LFA)-1 and very-late-antigen (VLA)-4 on EPCs binding to intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1, respectively. To finish incorporating the vessel, EPCs transmigrate mainly through β2 integrins binding to ICAM (E). For EPCs to differentiate, these cells must adhere to components of the extracellular matrix, such as fibronectin. Several molecules like VEGF and CXCL12 stimulate EPCs differentiation, which can be produced by MM PCs (F). Lastly, ECs secrete PDGF-B and recruit PDGFR-β^+ pericytes to stabilize the vasculature. MM PCs are also capable of recruiting pericytes through the same secretion (G).

EPCs reside in their stem cell niche characterized by high levels of CXCL12 (Caiado and Dias 2012). The main component controlling the detachment of stem cells from their niche seems to be matrix metalloproteinases (MMPs). These are a group of zinc-dependent endopeptidases with the capacity to degrade the ECM, thus contributing to the proliferation, invasion, metastasis, angiogenesis, and progression of cancer (Kessenbrock et al. 2015). More specifically, MMP-9 activation leads to progenitor cells, such as EPCs, leave their quiescent niche and move to a proliferative vascular niche (Heissig et al. 2002). Altered levels of MMP-9 are observed in MM. MMP-9 serum levels in MM patients were higher in Durie-Salmon stages II and III than in stage I (Alexandrakis et al. 2007). In fact, MMP-9 levels were higher when the murine MM PCs 5T33MM cells were co-cultured with BM ECs than with lung ECs (Van Valckenborgh et al. 2002). Interestingly, MMP-9 is secreted by MM PCs, whose upregulation is stimulated by BM ECs through hepatocyte growth factor (Van Valckenborgh et al. 2002, Vande Broek et al. 2004). Moreover, OCs can also secrete MMP-9 that will degrade the ECM and induce a vascular endothelial growth factor (VEGF) discharge (Ribatti et al. 2014). The contribution of MM BMSCs in cell detachment from the niche remains unclear, as it was observed that MM BMSCs secreted only MMP-1 and -2 but not MMP-9, which is the MMP most relevant in this process (Barillé et al. 1997). On the other hand, it seems that MMP-9 is not an indicator of bone disease in MM (Munemasa et al. 2007), which means that it could be relevant in some other event of the disease, such as neovascularization.

The recruitment of EPCs into the target location is a critical step in neovascularization. EPCs are mobilized and home to the location where they will incorporate the blood vessels and differentiate into ECs. A recently published study revealed that BM EPCs are mobilized to the location of MM PCs in the BM, which displacement is driven by the malignant cells (Moschetta et al. 2016). Many factors are involved in EPC mobilization such as CXCL12, VEGF, and also platelet-derived growth factor (PDGF)-B. The chemokine CXCL12 is produced by BMSCs (Nagasawa et al. 1994, de Nigris et al. 2012), BM ECs (Yun and Jo 2003), immature OBs and mesenchymal adipocytes (Lapidot and Kollet 2002). CXCL12 stimulates the motility of

cells with its receptors: CXCR4 and CXCR7 (de Nigris et al. 2012). CXCR4 is present in ECs, BM precursor cells and cancer cells such as MM PCs (Carr et al. 2006, Menu et al. 2006, de Nigris et al. 2012), while CXCR7 is characteristic of ECs and tumour cells (Asri et al. 2016). Both receptors are expressed by EPCs (Lu et al. 2015). The receptor CXCR4 seems to be essential for neovascularization, as its knockout supressed this vascular process. Moreover, the binding of CXCL12 to CXCR4 induces the release of VEGF on CXCR4-expressing human umbilical vein ECs, which may further promote the development of blood vessels (Salcedo et al. 1999). CXCL12 is also involved in the recruitment of MM PCs, in which research has focused. The chemokine was shown to be expressed and produced by MM PCs (Martin et al. 2006, Menu et al. 2006). Even though Hideshima and Anderson did not observe a very significant stimulation of MM PCs proliferation and migration induced by CXCL12, according to Menu *et al*. the blockade of CXCR4 resulted in a 50% decrease of the migration of MM PCs from a cell line to BMSCs, while the addition of CXCL12 resulted in an invasion increase of 4-fold (Hideshima and Anderson 2002, Menu et al. 2006). Martin *et al*. observed an increase in CXCL12 plasma levels from MGUS to MM patients, which may help perpetuate the angiogenic switch (Martin et al. 2006). The expression of both CXCL12 and CXCR4 by MM PCs would further stimulate this axis, through autocrine pathway, thus contributing to EPCs attraction. Until nowadays, MM research as only focused on chemoattraction applied to MM PCs. Although the connection between the CXCR4/CXCL12 axis and EPCs has been made, there seem to be no studies exploring it in MM.

VEGF is known for its ability to regulate vessel growth and permeability, and it is considered to be critical in vascular development (Yancopoulos et al. 2000). The role of VEGF and its receptors VEGFR-1 and -2 in the development of blood vessels has been widely studied in hematologic malignancies (Podar and Anderson 2008). VEGF has been shown to mobilize BM EPCs into circulation in neovascularization (Asahara et al. 1999, Li et al. 2006). The blockade of both VEGFR-1 and -2, which are present in haematopoietic cells and EPCs, resulted in the arrest of tumour neovascularization. This observation suggests not only that these cells participate in tumour neovascularization, but also that both VEGFR-1 and -2 participate in their recruitment (Lyden et al. 2001, Li et al. 2006). Regarding MM, VEGF plasma levels are higher in MM patients in Durie-Salmon stage III than in stages I and II (Valkovic et al. 2014). This is related with VEGF being produced by MM PCs (Kumar et al. 2003) to stimulate ECs proliferation and EPCs mobilization to the neovascularization site, since this factor detains this ability (Ria et al. 2008). It was also observed that the levels of cEPCs in MM were correlated with the rise of VEGF, which supports the importance of this growth factor in the trafficking of EPCs

(Bhaskar et al. 2012). In addition, specific genetic abnormalities on both VEGF and VEGFR-2 have also been linked with a more aggressive type of MM (Brito et al. 2014).

The recruited EPCs must adhere to the endothelium on the tumour site. This process is known to be promoted by several integrins, which are transmembrane adhesion proteins that mediate cell-ECM and cell-cell interactions. Every integrin has one α and one β subunit. Numerous integrins have been described to participate in several stages of EPC mobilization, but we choose to focus on their role in the adhesion of EPCs to the endothelium. β2 integrins are key players in the homing and adhesion of EPCs to sites of vascular remodelling. Besides β2 integrins, β1 integrins have also been demonstrated to participate in this process (Caiado and Dias 2012). Lymphocyte function-associated antigen (LFA)-1, which is part of the family of β2 integrins, and very late antigen (VLA)-4 or integrin α4β1 were found in high levels in MM, while in non-active MM and MGUS patients they were practically non-detectable. These molecules may support the interactions between MM PCs and the microvasculature (Vacca et al. 1995). CXCL12 was shown to modulate VLA-4 and, consequently, MM PCs adhesion (Sanz-Rodriguez et al. 2001). Interestingly, it was found that the contact of melanoma cells with ECs upregulated the expression of intercellular adhesion molecule (ICAM)-1, a ligand for LFA-1 (Zhang et al. 2014). Consequently, it is possible that MM malignant cells may also stimulate the expression of ICAM-1. Furthermore, co-culture of MM PCs with ECs upregulated the expression of vascular cell adhesion molecule (VCAM)-1, the ligand of VLA-4, on ECs (Wang et al. 2008). Therefore, MM PCs may upregulate ICAM-1 and VCAM-1 on ECs, which may promote the homing and adhesion of EPCs to ECs. Afterwards, transendothelial migration is mediated by β2 integrins for EPCs to integrate the site where vascular development is required (Chavakis et al. 2007).

Regarding EPCs differentiation, most studies have explored this process *in vitro*, but it may differ *in vivo*, due to interactions with other cells, the ECM and growth factors in their microenvironment (Hristov 2003). The adhesion to the ECM through fibronectins, one of the main fibrous ECM proteins, seems to be crucial for the differentiation of EPCs (Asahara et al. 1997, Frantz et al. 2010). Indeed, fibronectin stimulates VEGF-induced differentiation of EPCs through binding to the integrin α5β1 (Wijelath et al. 2004). Several authors have induced the differentiation of putative EPCs into ECs through the combination of VEGF with other growth factors such as basic fibroblast growth factor (bFGF) and insulin-like growth factor (IGF)-1 (Gehling et al. 2000, Quirici et al. 2001). Ria and colleagues hypothesized that through the expression of VEGF, bFGF and IGF, MM cells induce the differentiation of BM HSCs into MM ECs, thus contributing to neovascularization (Ria et al. 2008). BM levels of VEGF and bFGF are higher than the

respective PB levels in MM patients (Di Raimondo et al. 2000). In fact, individual PCs can secrete VEGF and bFGF (Kumar et al. 2004), stimulating EPCs differentiation. Furthermore, CXCL12 harbours the ability to induce EPCs proliferation and differentiation into ECs through the CXCR4 pathway, as it upregulates the expression of vWF, Tie-2 and VE-cadherin, all characteristic of ECs (Li et al. 2015). Since CXCL12 is also produced by MM PCs, these cells can also promote CXCL12-induced differentiation. Lastly, the regulation of the transcription factor HoxA is key for the acquisition of the endothelial phenotype of EPCs. Hence, the expression of endothelial genes for endothelial nitric oxide synthase, VEGFR-2 and VE-cadherin is promoted by *HoxA9* (Rössig et al. 2005).

The endothelium of blood vessels is supported by surrounding pericytes and vascular smooth muscle cells, as previously mentioned. PDGFs are main mitogens for cells of mesenchymal origin including smooth muscle cells and pericytes. These growth factors can have four polypeptide chains (A, B, C or D) that form homo- or heterodimers (AA, AB, BB, CC and DD). ECs produce PDGF-B, which recruits pericytes to sites of vascular remodelling, where pericytes play an important role in the maturation and stabilization of the vasculature through the binding of endothelial PDGF-B to the corresponding receptor, PDGFR-β, in pericytes (Fredriksson et al. 2004, Sá-Pereira et al. 2012, Hamdan et al. 2014). Hellström *et al*. demonstrated that PDGFR-β expression was greater in developing arteries and immature ECs (Hellström et al. 1999), which hints to the role that this receptor may have on EPCs. Later on, it was detected that EPCs express PDGFR-β (Guo et al. 2012). Interestingly, EPCs overexpressing PDGFR-β seem to have a higher capacity of re-endothelialisation (Wang et al. 2014). PDGFR-β was also observed to be expressed about 6 times more in a BM EPC cell line than in a brain capillary EC line. This was further correlated with PDGF-BB capacity to induce EPCs differentiation into smooth muscle cells (Miyata et al. 2005). Moreover, tumour-derived PDGFR-β^+ progenitor perivascular cells contribute to vasculature development and stability, as they were able to differentiate into pericytes in the presence of ECs (Song et al. 2005).Concerning the ligand, PDGF-BB stimulates EPC proliferation, migration and VEGF expression (Sufen et al. 2011). The PDGF-B signalling pathway was reported in tumours and its inhibition was associated with regression of tumour vessels (Sennino et al. 2007). Both MM PCs and BM ECs isolated from MM patients, at diagnosis and in relapse, expressed and secreted higher levels of PDGFR-β than the same cells isolated from MGUS patients (Coluccia et al. 2008). MGUS and SMM patients who evolve to MM also seem to have higher levels of PDGF-BB than patients who remain asymptomatic (Calcinotto et al. 2015). These data point to the significance of the PDGF

pathway in the progression of MGUS to MM. Furthermore, MM PCs are able to secrete PDGF-BB, which stimulates PDGFR-β on the same cells through autocrine pathway, but also in ECs through paracrine pathway. It was also confirmed that the PDGFR-β/PDGF-BB axis promotes MM PCs growth (Coluccia et al. 2008). The fact that MM PCs are able to secrete PDGFR-β/PDGF-B may also be connected with these cells ability to induce the proliferation and migration of EPCs. In addition, the production of PDGF-B in the tumour microenvironment was correlated with CXCL12 levels. It was shown that the disruption of CXCL12 decreases the PDGF-B expression in the tumour microenvironment, suggesting that the CXCL12/PDGF-B signalling pathway plays a key role in the differentiation of pericytes derived from the BM. Also, the blockade of the CXCR4/CXCL12 axis inhibits differentiation of BM cells in pericytes and their involvement in tumour vascular growth (Hamdan et al. 2014). On the other hand, PDGF-AB BM levels seem to be higher in MM patients than in the controls (Kara et al. 2006). Accordingly, PDGF-AB BM levels rise from Durie-Salmon stage I to II and III and are higher than in controls. Furthermore, PDGF-AB levels are higher in untreated MM patients in comparison to MM patients who received treatment. There was a correlation with these values and MVD (Tsirakis et al. 2012). Overall, there is evidence that in MM the stimulation of the PDGFR-β and ligands axis may lead to the recruitment pericytes, differentiation of progenitor cells and stabilization the vasculature, or for induction of EPC proliferation, migration, homing and even differentiation into other cells than those of the endothelial lineage. Even so, these findings need to be confirmed in MM, more specifically, the relation between PDGFR, the respective ligands, and EPCs.

The molecules mentioned have a central role in the recruitment and mobilization of EPCs in the BM. The levels displayed during several stages of MM vary, which suggests that they may be crucial to the disease development and progression. Clarifying the role of these molecules and others to be discovered in the recruitment and homing of EPCs in neovascularization is a subject that is far from being completed and needs more studies. Furthermore, these pathways may provide new targets for therapy in order to prevent the development of hematologic BM malignancies such as MM.

2. Goals

Despite the increasing number of studies concerning EPCs and MM, the variation of these cells in the BM either at different stages of disease or after treatment administration, as well as their relationship with PCs and with the expression of relevant signalling receptors, are still to be fully clarified. This prompted us to perform a retrospective and sequential study of MM patients in different disease stages, ranging from the pre-malignant to the condition achieved after treatment, in order to establish the temporal progression of EPCs levels and the relationship with PCs, $CXCR4^+$ and PDGFR-β^+ cells. To this end, the following specific objectives were outlined to: 1) develop a method to detect and quantify EPCs in archived BM smears relying on multiple immunofluorescence analysis of EPCs markers, 2) establish the temporal evolution of EPCs levels in sequential BM smears collected from (i) patients with MGUS that evolved to MM and from (ii) MM patients prior and after treatment, and determine the correlation with PCs levels, and 3) evaluate the cells positive for receptors CXCR4 and PDGFR-β and their relationship with EPCs. Ultimately, these studies will allow to establish the usefulness of EPCs as biomarkers of disease progression and response to therapy and their association with the signalling receptors CXCR4 and PDGFR-β.

3. Materials and Methods

3.1. Chemicals

Triton X-100, paraformaldehyde and glutaraldehyde were obtained from Merck Millipore (Darmstadt, Germany). Methanol, acetone, goat serum and rabbit serum were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). All other reagents were of analytical grade.

3.2. Patients

In this project, we performed a retrospective and longitudinal study profiting from the possibility of analysing BM smears archived at Instituto Português de Oncologia (IPO) Francisco Gentil, Lisbon, Portugal. The biologic material and the corresponding clinical information were provided by Dra. Margarida Silveira, Director of the Clinical Pathology Center of IPO, under a protocol established between IPO and Faculty of Pharmacy, Universidade de Lisboa. The BM smears were collected between December 2006 and May 2016. These were attained from BM aspirations from the posterior iliac crest and the smears were fixed by air-drying by standard procedures. May-Grünwald-Giemsa staining was performed in all BM smears to quantify the amount of PCs based on their characteristic morphology with an eccentric nucleus and large cytoplasm. The patients' clinicopathological data such as sex, age, BM PCs %, serum M protein spike, FLC, and levels of β2-microglobulin, albumin, haemoglobin and creatinine were recorded.

MGUS and healthy smears were incredibly sparse to find, since BM biopsies are not usually done in patients with no symptoms. Even so, we were able to get a reasonable amount of MGUS samples. A total of 35 patients was analysed: 6 patients in MGUS that evolved to MM, and 29 patients with MM that received treatment, of which 23 achieved complete REM (cREM), 3 achieved partial REM (pREM) and 3 did not achieve REM (non-REM). The number of patients and the number of smears available per patient and disease stage determined the immunofluorescence analysis per groups of patients that was possible to perform (Fig. 5). The parameters experimentally analysed in each group of patients are indicated in Table 2. Clinicopathological parameters and PC content of each patient are depicted in Table 3. The average age was 59.5 ± 9.6 years old, ranging from 40 to 89 years. While 22 patients were men, 13 were female. Serum M protein peak and FLC were analysed, revealing 20 patients with M protein IgG, 6 with IgA, 2 with IgD and 7 with FLC. ISS staging was used to characterize the stages of MM. ISS I was defined with serum β2-microglobulin as less than 3.5 mg/L and albumin level as 3.5 g/dL or greater, and ISS III with serum β2-microglobulin as 5.5 mg/L or

greater. ISS II was defined as neither stage I nor III; in other words, serum β2-microglobulin between 3.5 and 5.5 mg/L with any albumin level, or albumin below 3.5 g/dL while the β2-microglobulin is less than 3.5 mg/L. The average of serum β2-microglobulin in the patients in MGUS was 6.2 ± 6.5, 3.9 ± 1.5 in MM and 3.8 ± 2.7 mg/L after treatment administration. Concerning the albumin levels, these displayed an average of 3.9 ± 0.3 in MGUS, 4.3 ± 0.1 in MM and 4.1 ± 0.3 g/dL with therapy. Although the evaluation of treatment response in MM is dependent on M protein serum levels, FLC ratio and BM PCs % (Kumar et al. 2016), only complete data about BM PCs % was provided. Therefore, patients that received treatment were classified and divided in the following groups according to BM PCs: 1) non-REM, the patients with PCs≥10%, 2) pREM, the patients with 5≤ PCs<10%, and 3) cREM the patients with PCs<5%. Serum haemoglobin and creatinine were also evaluated. The average of serum haemoglobin in the patients in MGUS was 11.6 ± 1.7, 11.4 ± 2.2 in MM and 12.0 ± 1.93 g/dL after treatment administration. On the other hand, the patients displayed serum creatinine with an average of 2.1 ± 2.4 in MGUS, 1.7 ± 1.7 in MM and 1.2 ± 1.1 mg/dL in treated MM.

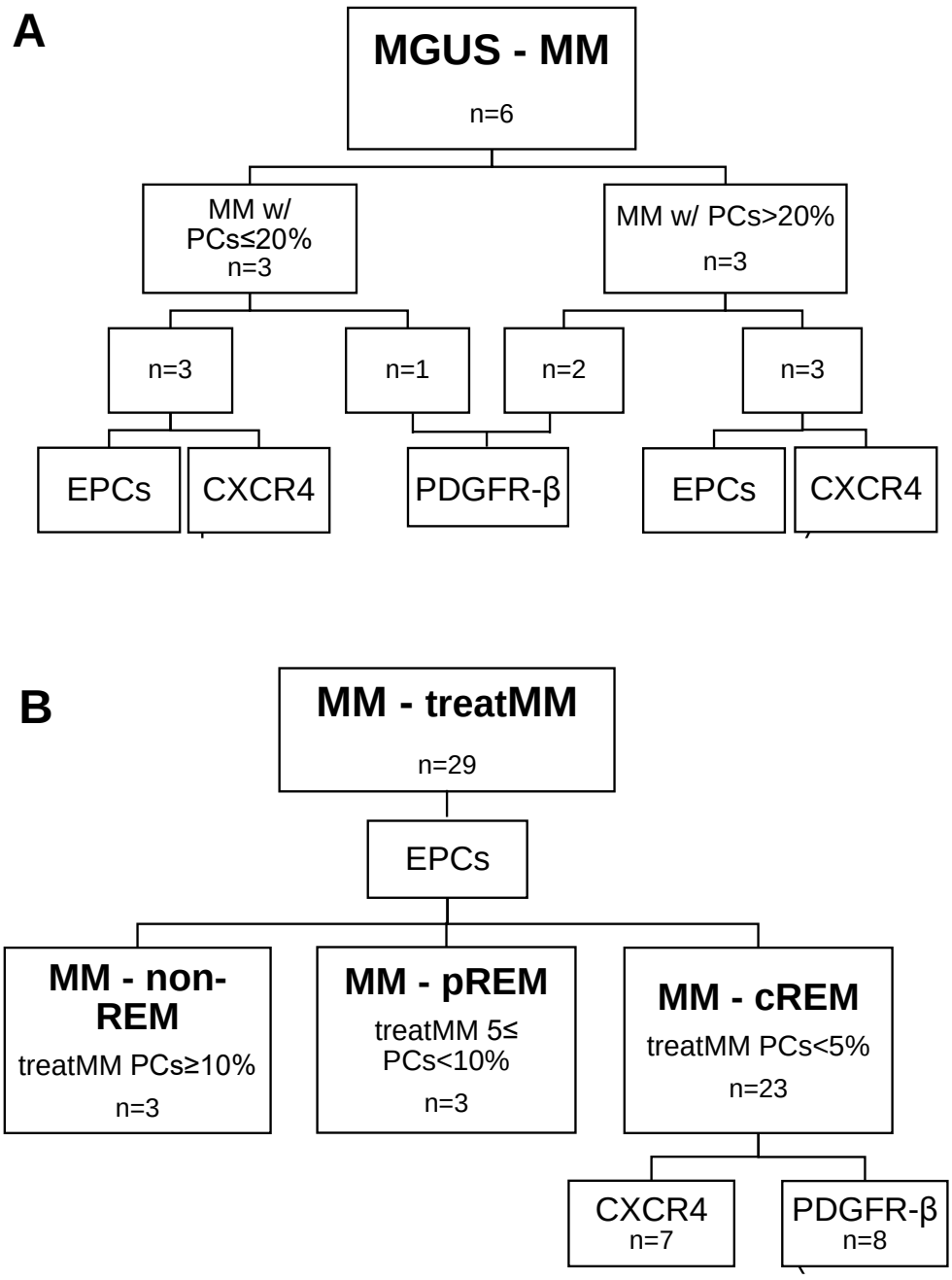

Figure 5: Schematic representation of the organization of the groups of patients. Six patients evolved from monoclonal gammopathy of undetermined significance (MGUS) to multiple myeloma (MM), in which 3 displayed plasma cells (PCs)≤20% in MM and the remaining 3 had PCs>20% in MM. In 1 of the 3 patients with PCs≤20% and 2 from the 3 patients with PCs>20% the levels of platelet-derived growth factor receptor (PDGFR)-β were assessed. Both endothelial progenitor cells (EPCs) and C-X-C motif chemokine receptor (CXCR)4 levels were analysed in every 6 MGUS-MM patients. A total of 29 patients with MM received treatment (treatMM), whose EPCs levels were evaluated. Out of this group, 3 patients did not achieve remission (non-REM), 3 achieved partial remission (pREM) and 23 achieved complete remission (cREM). In the 23 MM-cREM patients, 7 patients had their CXCR4 levels evaluated and 8 had their PDGFR-β levels assessed.

Table 2: Parameters experimentally analysed by immunofluorescence in each group of patients.

Clinical condition	MGUS	MM	TreatMM	MGUS	MM	TreatMM	MGUS	MM	TreatMM
Case	**EPCs**			**CXCR4**			**PDGFR-β**		
1	✓	✓	-	✓	✓	-	✓	✓	-
2	✓	✓	-	✓	✓	-	✓	✓	-
3	✓	✓	-	✓	✓	-	-	-	-
4	✓	✓	-	✓	✓	-	✓	✓	-
5	✓	✓	-	✓	✓	-	-	-	-
6	✓	✓	-	✓	✓	-	-	-	-
7	-	✓	✓	-	-	-	-	-	-
8	-	✓	✓	-	-	-	-	-	-
9	-	✓	✓	-	-	-	-	-	-
10	-	✓	✓	-	-	-	-	-	-
11	-	✓	✓	-	-	-	-	-	-
12	-	✓	✓	-	-	-	-	-	-
13	-	✓	✓	-	-	-	-	-	-
14	-	✓	✓	-	-	-	-	-	-
15	-	✓	✓	-	-	-	-	-	-
16	-	✓	✓	-	-	-	-	-	-
17	-	✓	✓	-	-	-	-	-	-
18	-	✓	✓	-	-	-	-	-	-
19	-	✓	✓	-	-	-	-	-	-
20	-	✓	✓	-	-	-	-	-	-
21	-	✓	✓	-	✓	✓	-	-	-
22	-	✓	✓	-	✓	✓	-	-	-
23	-	✓	✓	-	✓	✓	-	-	-
24	-	✓	✓	-	✓	✓	-	-	-
25	-	✓	✓	-	✓	✓	-	-	-
26	-	✓	✓	-	✓	✓	-	-	-
27	-	✓	✓	-	✓	✓	-	-	-
28	-	✓	✓	-	-	-	-	✓	✓
29	-	✓	✓	-	-	-	-	✓	✓
30	-	✓	✓	-	-	-	-	✓	✓
31	-	✓	✓	-	-	-	-	✓	✓
32	-	✓	✓	-	-	-	-	✓	✓
33	-	✓	✓	-	-	-	-	✓	✓
34	-	✓	✓	-	-	-	-	✓	✓
35	-	✓	✓	-	-	-	-	✓	✓

CXCR4, C-X-C motif chemokine receptor 4; EPCs, endothelial progenitor cells; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; PDGFR-β, platelet-derived growth factor receptor β; treatMM, treated multiple myeloma.

Table 3: Clinicopathological parameters of the 35 patients analysed

					β2-microglobulin (mg/L)			Albumin (g/dL)			BM PCs (%)			Haemoglobin (g/dL)			Creatinine (mg/dL)		
Case	**Sex**	**Age**	**M protein/ FLC**	**ISS**	**MGUS**	**MM**	**Treat MM**	**MGUS**	**MM**	**Treat MM**	**MGUS**	**MM**	**Treat MM**	**MGUS**	**MM**	**Treat MM**	**MGUS**	**MM**	**Treat MM**
1	M	54	IgA K	?	1.5	-	-	4.2	4.2	-	7.0	13.0	-	15.0	15.3	-	0.9	0.7	-
2	M	60	IgG K	?	2.8	-	-	-	-	-	4.6	20.0	-	10.5	10.6	-	1.2	2.3	-
3	M	89	IgA K	II	4.5	5.0	-	4.2	4.4	-	5.0	10.0	-	10.3	10.3	-	1.5	1.5	-
4	F	66	FLC ƛ	?	19.1	-	-	3.8	4.2	-	6.0	32.6	-	10.8	11.0	-	7.0	2.5	-
5	M	67	IgG K	?	3.2	-	-	-	-	-	6.4	28.2	-	11.6	8.7	-	0.9	-	-
6	M	76	FLC K	?	6.2	2.8	-	3.6	-	-	6.0	36.8	-	11.2	14.5	-	1.3	1.4	-
7	M	62	IgG K	III	-	21.2	-	-	2.8	3.8	-	60.0	2.6	-	10.5	11.8	-	5.9	2.0
8	F	64	IgG ƛ	?	-	?	-	-	?	-	-	31.0	3.0	-	10.1	10.9	-	0.8	0.8
9	M	52	IgG K	II	-	2.4	1.8	-	3.3	4.2	-	27.2	4.0	-	15.3	13.4	-	0.9	0.4
10	F	56	IgD ƛ	II	-	5.2	-	-	3.4	-	-	45.8	1.0	-	10.2	12.2	-	0.7	0.7
11	F	59	IgG ƛ	III	-	9.1	-	-	-	-	-	90.0	1.0	-	8.6	10.6	-	1.6	1.1
12	M	65	IgG K	II	-	3.5	-	-	4.3	-	-	32.0	3.0	-	13.2	11.4	-	0.8	0.7
13	M	40	IgG K	I	-	2.6	-	-	5.0	3.6	-	78.8	0.6	-	14.2	13.4	-	0.9	0.8
14	F	62	IgG ƛ + FLC	I	-	2.6	2.4	-	4.1	4.3	-	22.8	1.4	-	11.1	10.2	-	1.1	0.8
15	M	72	IgG K	III	-	45.0	10.3	-	2.8	4.1	-	27.6	8.0	-	8.7	10.3	-	8.4	6.6
16	M	66	FLC K	I	-	2.9	2.5	-	4.5	4.0	-	27.0	6.6	-	12.0	15.0	-	0.7	0.7
17	M	66	FLC K	I	-	2.5	2.9	-	4.0	4.1	-	27.0	6.6	-	15.1	12.0	-	0.7	0.7
18	M	58	IgG K	I	-	2.7	2.7	-	4.7	4.6	-	31.0	11.4	-	13.4	13.2	-	1.2	1.1
19	M	64	IgG ƛ	II	-	4.3	-	-	4.3	4.3	-	16.0	11.2	-	10.4	9.9	-	0.9	1.1
20	F	60	IgG ƛ	I	-	3.4	-	-	3.8	-	-	48.8	20.0	-	10.8	12.4	-	0.7	-
21	M	55	IgA K	II	-	3.8	-	-	-	3.9	-	22.8	0.2	-	13.7	13.6	-	0.7	0.6
22	F	56	FLC K	III	-	11.4	5.8	-	4.1	4.3	-	39.0	1.0	-	10.0	10.7	-	3.6	1.9
23	M	57	IgG ƛ	I	-	2.8	-	-	3.8	4.6	-	27.8	2.0	-	9.0	14.2	-	0.9	0.8
24	M	51	IgA K	III	-	14.8	1.8	-	3.4	3.9	-	70.0	0.5	-	7.7	14.2	-	1.6	1.0
25	F	57	IgD ƛ	II	-	5.2	-	-	3.4	-	-	45.8	3.8	-	10.2	13.2	-	0.7	0.7
26	M	41	IgA K	I	-	3.3	-	-	3.5	4.5	-	43.2	0.4	-	11.9	11.7	-	1.1	1.0
27	F	56	FLC K	III	-	11.4	5.8	-	4.0	-	-	18.4	1.0	-	10.0	10.7	-	3.6	2.0
28	F	58	IgG K	I	-	1.7	-	-	4.0	-	-	19.0	0.6	-	12.2	12.9	-	0.7	0.6
29	F	53	IgA ƛ	?	-	-	-	-	3.0	3.8	-	71.6	2.8	-	8.0	8.9	-	4.4	1.8
30	M	46	FLC ƛ	I	-	2.2	-	-	4.5	4.3	-	82.7	2.0	-	11.2	15.4	-	0.8	0.7
31	M	62	IgG K	?	-	-	-	-	4.7	-	-	40.0	0.2	-	15.8	13.4	-	1.3	0.9
32	M	60	IgG K	?	-	-	-	-	-	-	-	17.0	3.0	-	13.3	12.6	-	0.7	0.7
33	F	69	IgG ƛ	II	-	3.7	-	-	3.6	-	-	25.6	1.0	-	11.3	6.2	-	0.7	0.6
34	M	61	IgG ƛ	II	-	5.0	-	-	5.0	3.7	-	49.0	0.6	-	9.7	12.0	-	1.4	0.8
35	F	43	IgG ƛ	I	-	2.6	1.8	-	3.5	4.7	-	19.8	2.0	-	11.5	13.0	-	0.6	0.7

F, female; FLC, free light chain; M, male; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; M protein, monoclonal protein; treatMM, treated multiple myeloma; ?, inconclusive information; -, unavailable information.

3.3. Development of a multiple immunofluorescence method for assessment of endothelial progenitor cells levels in bone marrow smears

The absence of a method to determine EPCs levels in archived BM smears, in conjunction with the lack of a specific marker of these progenitor cells of the endothelial lineage, prompted us to develop a novel method. We relied on multiple immunofluorescence analysis of CD34, CD133 and VEGFR-2, markers commonly used to detect EPCs (Massa et al. 2005, Caiado et al. 2011, Blix et al. 2015). CD34 is a transmembrane phosphoglycoprotein expressed by HSCs and ECs of small vessels (Fadini et al. 2008, Shi and VandeBerg 2015). CD133 is a transmembrane glycoprotein expressed solely by haematopoietic stem and progenitor cells, such as EPCs (Shmelkov et al. 2005), whose expression decreases during EPCs differentiation (Hristov 2003). VEGFR-2 is a transmembrane tyrosine kinase receptor involved in the formation of blood and lymphatic vessels, expressed by cells of the endothelial lineage (Fadini et al. 2008, Guo et al. 2010). To develop and optimize the multiple immunofluorescence assay, numerous conditions and steps were tested, namely several solutions for the fixation, permeabilization and blocking steps, in addition to dilutions of the primary and secondary antibodies (Supplementary table 1). The fixation step was the hardest to optimize. Fixatives such as glutaraldehyde, paraformaldehyde, methanol, acetone and ethanol were tested in various concentrations and incubation times, but they failed to preserve the smear integrity and often produced high autofluorescence. The final fixation solution had to be mixed with the permeabilization solution. After the optimal solutions were found, different combinations of double and triple labelling were also tested (Supplementary table 2), until the optimal method was established.

The optimized multi-labelling immunofluorescence method for EPCs is schematically represented in Figure 6 and the experimental conditions are summarized in Table 4. BM smears were incubated with a 75% methanol/25% acetone/0.01% Triton X-100 solution (20 minutes at -20ºC) for fixation and permeabilization. The triple-labelling demanded a two-steps procedure, wherein smears were blocked and incubated with two primary antibodies and the corresponding secondary antibodies, followed by a second blocking step and incubation with the third primary antibody and the respective secondary antibody. Blocking was performed by incubation with serum of the same species of the secondary antibody. All steps were followed by washes with phosphate buffered saline (PBS) three times for 5 minutes, except for the step between the blocking solution and the incubation of the primary antibodies. For the first step, blocking was performed with a 10% goat serum and 1% bovine serum albumin (BSA) solution in PBS; smears were then incubated with a solution containing the primary antibodies CD133

and VEGFR-2 in the goat serum blocking solution, followed by the respective secondary antibodies, Alexa Fluor® 555 and 647, diluted in the same blocking solution. The second step was performed by blocking with 10% rabbit serum and 1% BSA solution in PBS, followed by incubation with the primary antibody CD34, and then by incubation with the respective secondary antibody Alexa Fluor® 488, both diluted in the rabbit serum blocking solution. Negative controls with omission of the primary antibody were performed to assure the specificity of the labelling. For nuclear labelling, smears were incubated with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies, Carlsbad, California, USA) diluted in PBS (1:1000) for 3 minutes. The slides were rinsed three times with PBS afterwards. Washing and rising steps were performed in a 1L beaker. The slides were mounted using the mounting medium SlowFade® Diamond Antifade Mountant (ThermoFisher Scientific, Waltham, Massachusetts, USA) and a coverglass 23.8 x 60 mm, 0.13-0.17 mm thick (Medite, Medizintechnik).

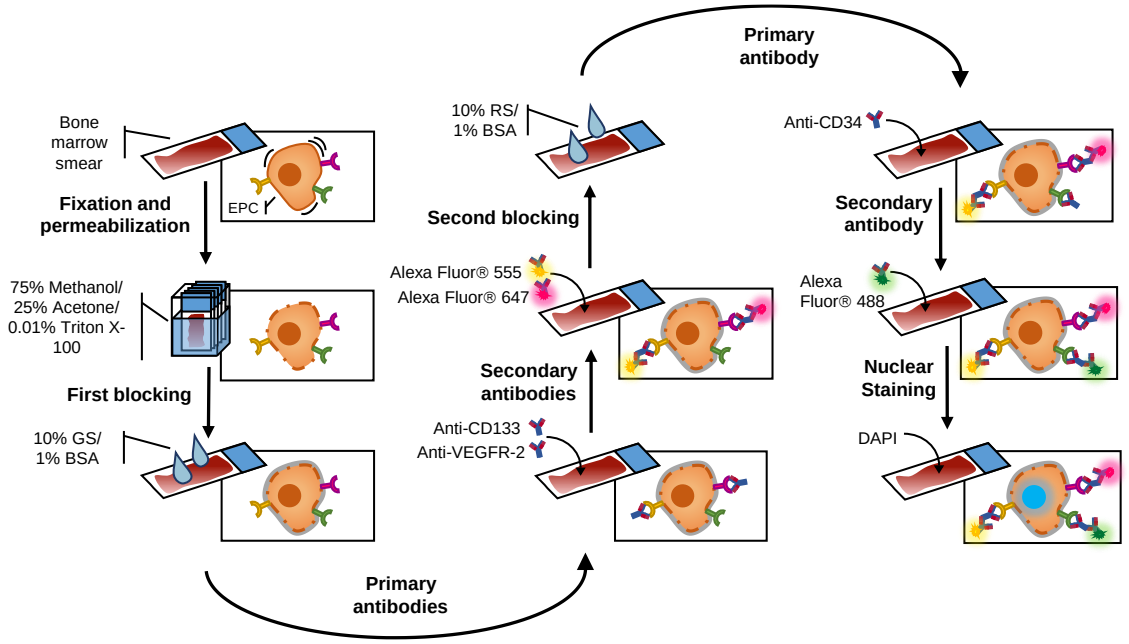

Figure 6: Schematic representation of the optimized multiple immunofluorescence method for endothelial progenitor cells (EPCs) detection in bone marrow (BM) smears. The slides with the BM smears are placed in a staining dish with a solution of 75% methanol/25% acetone/0.01% Triton X-100 for fixation and permeabilization. The slides are then incubated with the first blocking solution [10% goat serum (GS),1% bovine serum albumin (BSA)] followed by incubation with the primary antibodies cluster of differentiation (CD)133 and vascular endothelial growth factor receptor-2 (VEGFR-2), and the respective secondary antibodies Alexa Fluor® 555 and 647, both diluted in the first blocking solution. Next, the second blocking solution [10% rabbit serum (RS), 1% BSA] is applied, followed by the incubation with the third primary antibody CD34 and the third secondary antibody Alexa Fluor® 488. Finally, DAPI (4',6-diamidino-2-phenylindole) is used to label the nuclei.

Table 4: Summary of the antibodies and experimental conditions used for multiple immunofluorescence analysis.

	Marker	Blocking	Primary Antibody	Secondary Antibody
First step of incubations: combination of antibodies	CD133	10% GS and 1% BSA in PBS, 1 h, RT	CD133, rabbit Pc, 1:100 in BS, ON, 4ºC ThermoFisher Scientific, #PA5-38014	Goat Alexa Fluor® 555 anti-rabbit, 1:500 in BS, 1 h, RT ThermoFisher Scientific, #A-21428
	VEGFR-2	10% GS and 1% BSA in PBS, 1 h, RT	VEGFR-2, mouse Mc, 1:200 in BS, ON, 4ºC ThermoFisher Scientific, #MA5-15556	Goat Alexa Fluor® 647 anti-mouse, 1:500 in BS, 1 h, RT ThermoFisher Scientific, #A-21235
Second step of incubation	CD34	10% RS and 1% BSA in PBS, 1 h, RT	CD34, rat Mc, 1:100 in BS, ON, 4ºC ThermoFisher Scientific, #MA1-22646	Rabbit Alexa Fluor® 488 anti-rat, 1:250 in BS, 1 h, RT ThermoFisher Scientific, #A-21210

BS, blocking solution; CD, cluster of differentiation; GS, goat serum; Mc, monoclonal; ON, overnight; PBS, phosphate buffered saline; Pc, polyclonal; RS, rabbit serum; RT, room temperature; VEGFR-2, vascular endothelial growth factor receptor 2; #, catalogue reference.

3.4. Single immunofluorescence of CXCR4 and PDGFR-β

Immunofluorescence analysis of CXCR4 and PDGFR-β was also performed to identify cells positive for each of these receptors in BM smears. Fixation and permeabilization, as well as washings, were performed as described for the multiple labelling. Blocking was performed with a 10% goat serum and 1% BSA solution in PBS. Incubations with the primary antibodies rabbit polyclonal anti-CXCR4 (ThermoFisher Scientific, #PA3-305) in blocking solution (1:100), or rabbit monoclonal anti-PDGFR-β (ThermoFisher Scientific, #MA5-15143) in blocking solution (1:100) were performed overnight at 4ºC. This was followed by incubation with the secondary antibody goat Alexa Fluor® 555 anti-rabbit (ThermoFisher Scientific, #A-21428) in blocking solution (1:500) for 1 hour at room temperature. Negative controls with the omission of the primary antibodies were performed to exclude nonspecific binding or cross-reactivity.

3.5. Confocal immunofluorescence and bright-field microscopy

The BM smears were visualized in microscopes available at the Microscopy Lab of the Faculty of Sciences, Universidade de Lisboa that were used thanks to a collaboration with Prof. Rui Malhó, Head of the facility. The May-Grünwald-Giemsa stained-smears were observed in a bright field microscope Olympus BX51 and photographed with an integrated digital camera Olympus DP50. The immunofluorescence samples were observed in the high-resolution spectral confocal system Leica TCS SPE, based on a Leica DMI4000B microscope, equipped with 3 lasers and one transmitted light detector (mercury metal halide bulb) with 3 filter sets. The 488

nm laser was used for the imaging of the primary antibody CD34 labelling, the 532 nm laser for the primary antibody CD133 and the 632 nm laser for the primary antibody VEGFR-2. The filter set with excitation 330-385 nm and emission of >420 nm was used to visualize the DAPI labelling. Confocal images were taken with the microscope's detector. Due to the absence of an ultraviolet laser, DAPI labelling was not imaged in confocal mode, but on the fluorescence mode with the specific fluorescence filter for blue fluorophores. For image recording of the DAPI labelling, an Olympus C5060 camera with an Olympus C5060-ADU adapter was used. Confocal microscopy images were opened and merged in ImageJ 1.29x software (National Institutes of Health, USA) with LOCI plugin. These were merged with DAPI images using Photoshop Elements 13 (Adobe Systems, San Jose, California, USA).

Each experimental condition was observed and quantified in 10 random fields (400x magnification) per smear, for cell and nuclei count. The population of EPCs was considered as the positive cells for the 3 markers CD133, VEGFR-2 and CD34, identified in merged images. $CXCR4^+$ cells and PDGFR-β^+ cells were considered as the cells presenting a clearly visible nucleus that were positive for the respective receptor and did not display PC morphology. The count of the number of positive EPCs, $CXCR4^+$ cells and PDGFR-β^+ cells and the total number of nuclei per field was used to calculate the % of each cell type per field. Mean values were calculated for each slide, which were used to determine the mean value for each disease stage.

3.6. Statistics

The data is presented as mean ± standard deviation. GraphPad Prism 5 for Windows (GraphPad Software, San Diego, California, USA) was used for statistical analysis. Student's *t* test (two-tailed) was used to compare the same variables amongst different stages. Correlation between the different variables and numerical clinicopathological parameters in the same stage was evaluated using Pearson's r. For n=3, correlations were calculated with GraphPad InStat (GraphPad Software, San Diego, California, USA). P values of less than 0.05 were regarded as statistically significant.

4. Results

4.1. Identification of endothelial progenitor cells in bone marrow smears by multiple labelling immunofluorescence analysis

With the rising interest in EPCs in the last decades, we aimed at developing a novel method for detection of these cells in BM smears considering that EPCs are produced in the BM and that their analysis in smears allows for the study of archived material and, thus, the realization of retrospective studies. Since there is no single and specific marker of EPCs, we chose to use the markers CD34, CD133 and VEGFR-2 to detect EPCs. As shown in Figure 7, CD34, CD133 and VEGFR-2 exhibited strong signals, with a low background signal due to autofluorescence, whereas negative controls showed no labelling. By merging the different images it was possible to identify the triple positive cells with a round morphology as EPCs. Moreover, labelling the nuclei with DAPI renders possible to determine the % of EPCs. Thus, this multiple labelling immunofluorescence method allows the identification and quantification of EPCs in BM smears.

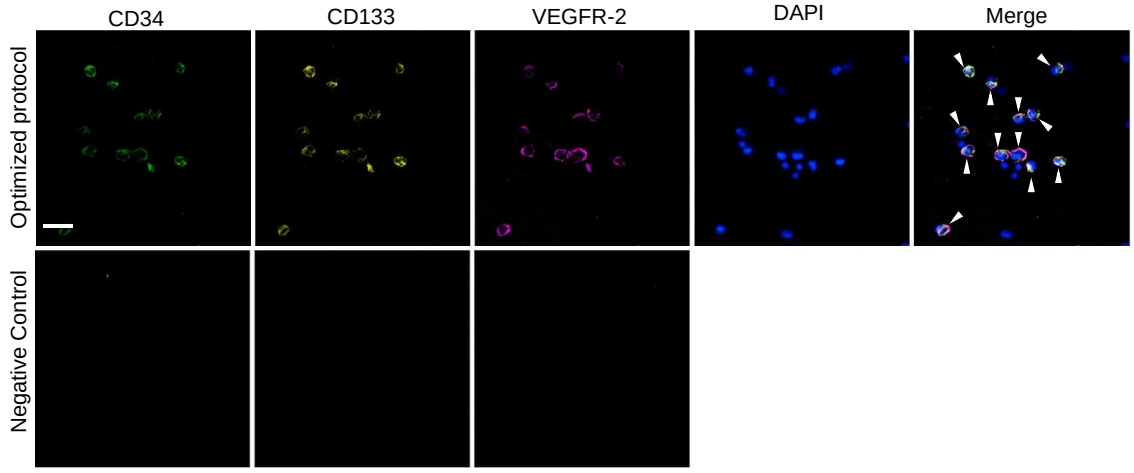

Figure 7: Identification of endothelial progenitor cells (EPCs) in bone marrow (BM) smears by triple-labelling immunofluorescence analysis of cluster of differentiation (CD)34, CD133 and vascular endothelial growth factor receptor (VEGFR)-2, with nuclei visualization by 4',6-diamidino-2-phenylindole (DAPI). BM smears of multiple myeloma patients were processed for immunofluorescence analysis and EPCs are pointed by arrowheads (first row); negative controls of the mentioned antigens are shown (second row). Representative images are shown. Scale bar: 20 µm.

4.2. Endothelial progenitor cells levels according to MM progression and response to therapy

Based on previous studies hinting to the potential of EPCs as a biomarker for MGUS progression to MM and for the response to MM treatment (Zhang et al. 2005, Udi et al. 2011, Bhaskar et al. 2012), and the absence of studies exploring this evolution in

the same patients, we investigated the temporal evolution of the % of EPCs in the BM, in sequentially collected samples of specific patients. Thus, using triple immunofluorescence plus DAPI labelling, cells simultaneously positive for CD34, CD133 and VEGFR-2 were identified (Fig. 8A-B) and their % relatively to the number of nuclei labelled with DAPI was calculated (Fig. 8C-F). Moreover, BM PCs % of each patient were analysed by May-Grünwald-Giemsa staining (Fig. 8A-B) and their quantification was performed (Fig. 8C, 8E). Since MM is a PC proliferative disorder, the amount of PCs in the BM is crucial for diagnosis and evaluation of response to therapy (Kurtin 2013). As expected, the evolution of MGUS to MM was characterized by a significant increase in PCs levels, from 5.8% to 23.4% ($p<0.01$). In contrast, a trend to a reduction was observed in EPCs levels, which decreased from 36.5% in MGUS to 25.4% in MM, though the difference did not achieve significance (Fig. 8C). An in-depth analysis of EPCs levels was performed as a function of BM levels of MM PCs, considered as ≤20% or >20%. Interestingly, EPCs levels decreased significantly from 49.0% in MGUS to 24.8% in MM patients with the highest BM infiltration ($p<0.01$), whereas no significant changes were observed in the patients with the lowest BM infiltration (23.9% in MGUS and 26.0% in MM) (Fig. 8D). This indicates that EPCs levels are higher in MGUS patients that progress to a more severe MM than in patients who progress to a lesser severe MM condition.

As previously explained, we considered as non-REM the patients with PCs≥10%, as pREM the patients with 5%≤PCs<10% and as cREM those with PCs<5%. Interestingly, while PCs levels decreased from 39.0 to 2.9% from MM to REM (pREM and cREM patients), EPCs levels increased significantly from 19.4 to 37.9% ($p<0.001$, Fig. 8E). Next, we evaluated separately EPCs levels of patients who had not achieved REM after receiving therapy, who achieved pREM and cREM (Fig. 8F). There was an increase in EPCs levels after the treatment administration in the patients who achieved pREM (10.2 to 43.1%, $p<0.05$) and in the patients that achieved cREM (19.3 to 36.8%, $p<0.001$), whereas in non-REM patients EPCs levels remained unchanged. EPCs levels in MM patients that did not achieve REM were higher (31.0%) in comparison to patients who reached pREM and cREM (10.2 and 19.3%, respectively).

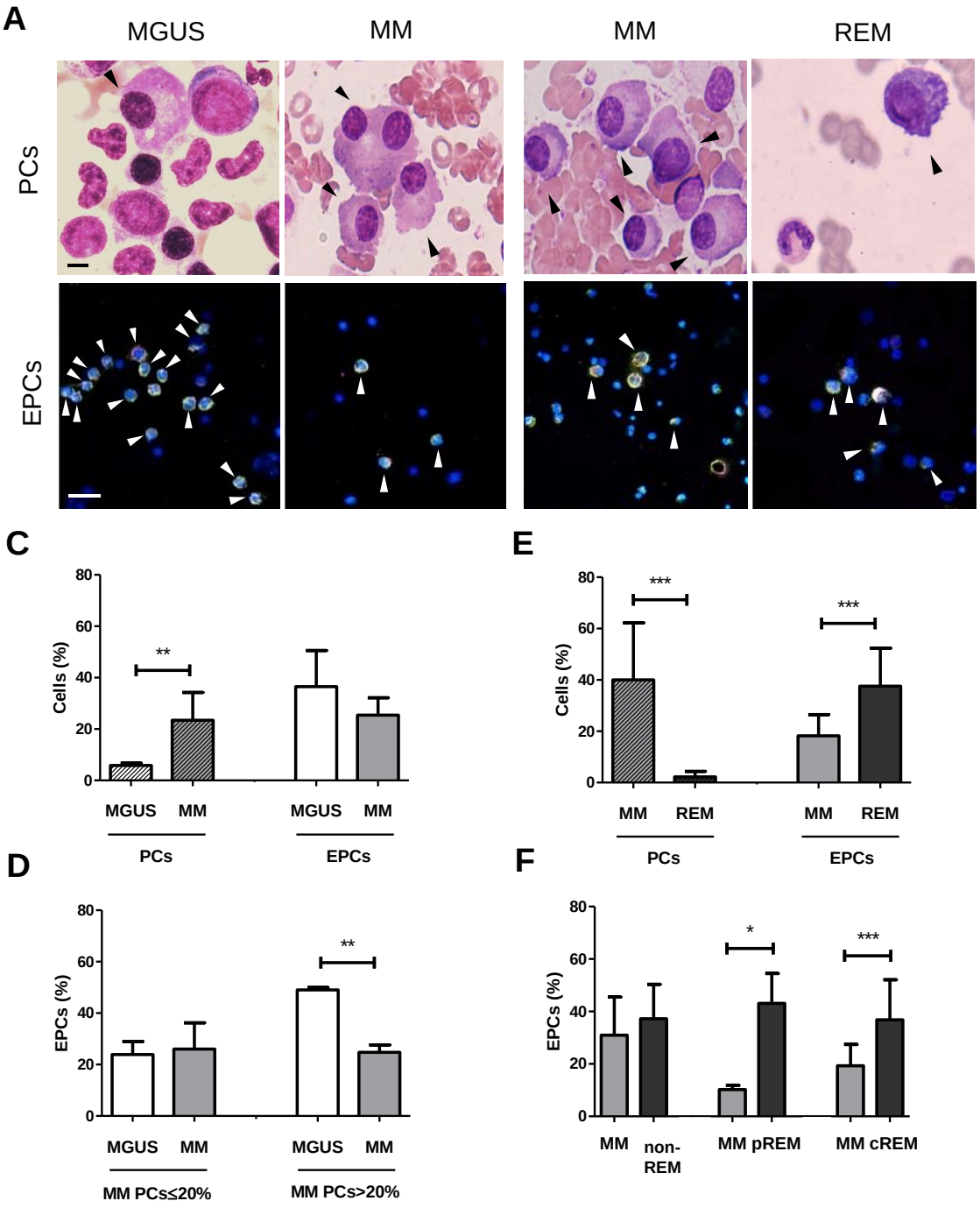

Figure 8: Plasma cells (PCs) and endothelial progenitor cells (EPCs) in the bone marrow (BM) of patients with monoclonal gammopathy of undetermined significance (MGUS), multiple myeloma (MM) and patients after treatment. Staining of BM smears with May-Grünwald-Giemsa allowed the visualization of PCs (A-B upper panels, black arrowheads), while triple immunofluorescence of cluster of differentiation (CD)34, CD133 and vascular endothelial growth factor receptor-2 allowed visualization of EPCs (A-B, lower panels, white arrowheads). Quantitative analysis of percentage (%) of PCs and EPCs in sequentially collected BM samples of MGUS patients that evolved to MM (n=6) (C), which were further analysed accordingly with MM PCs≤20% (n=3) and MM PCs>20% (n=3) (D). The images in panel A correspond to patients with MGUS who evolved to MM with PCs>20%. Quantitative analysis of the % of PCs and EPCs in sequential samples of MM patients that entered the remission (REM) stage (partial remission [pREM] and complete remission [cREM]) (n=26) (E), which representative

images are in panel B. % of EPCs in MM patients who after treatment did not achieved REM (non-REM) (n=3), who achieved pREM (n=3) and patients with cREM (n=23) (F). Data is shown as mean + standard deviation, *p<0.05, **p<0.01, ***p<0.001. Black scale bar: 50 µm. White scale bar: 20 µm.

4.3. Alterations in CXCR4$^+$ cells percentage from MGUS to MM and from MM to cREM

CXCR4 is the receptor of CXCL12, a key regulator of retention, migration, homing and mobilization of EPCs by chemoattraction (Menu et al. 2006). With the purpose of evaluating the temporal evolution of CXCR4$^+$ cells and if the expression of CXCR4 could be attributed mainly to EPCs, we determined the % of positive cells by immunofluorescence analysis, excluding from the quantification the positive cells with an eccentric nuclei, characteristic of PCs (Fig. 9A-B). Analysis of the results showed that the patients who evolved from MGUS to MM with PCs>20% in MM, presented a decrease in CXCR4$^+$ cells from 55.1% in the pre-malignant phase to 27.0% in the active disease ($p<0.05$, Fig. 9C). The group of patients with less severe disease, who had PCs≤20% in MM, showed no statistical significance (not shown). On the other hand, CXCR4$^+$ cells levels increased from 27.0% in MM to 38.6% in patients that achieved cREM after treatment (Fig. 9D). Interestingly, these patterns resembled the ones of EPCs throughout MM stages, with significant lower levels in MM as compared to MGUS. Thus, it is conceivable that EPCs are associated with the expression of CXCR4.

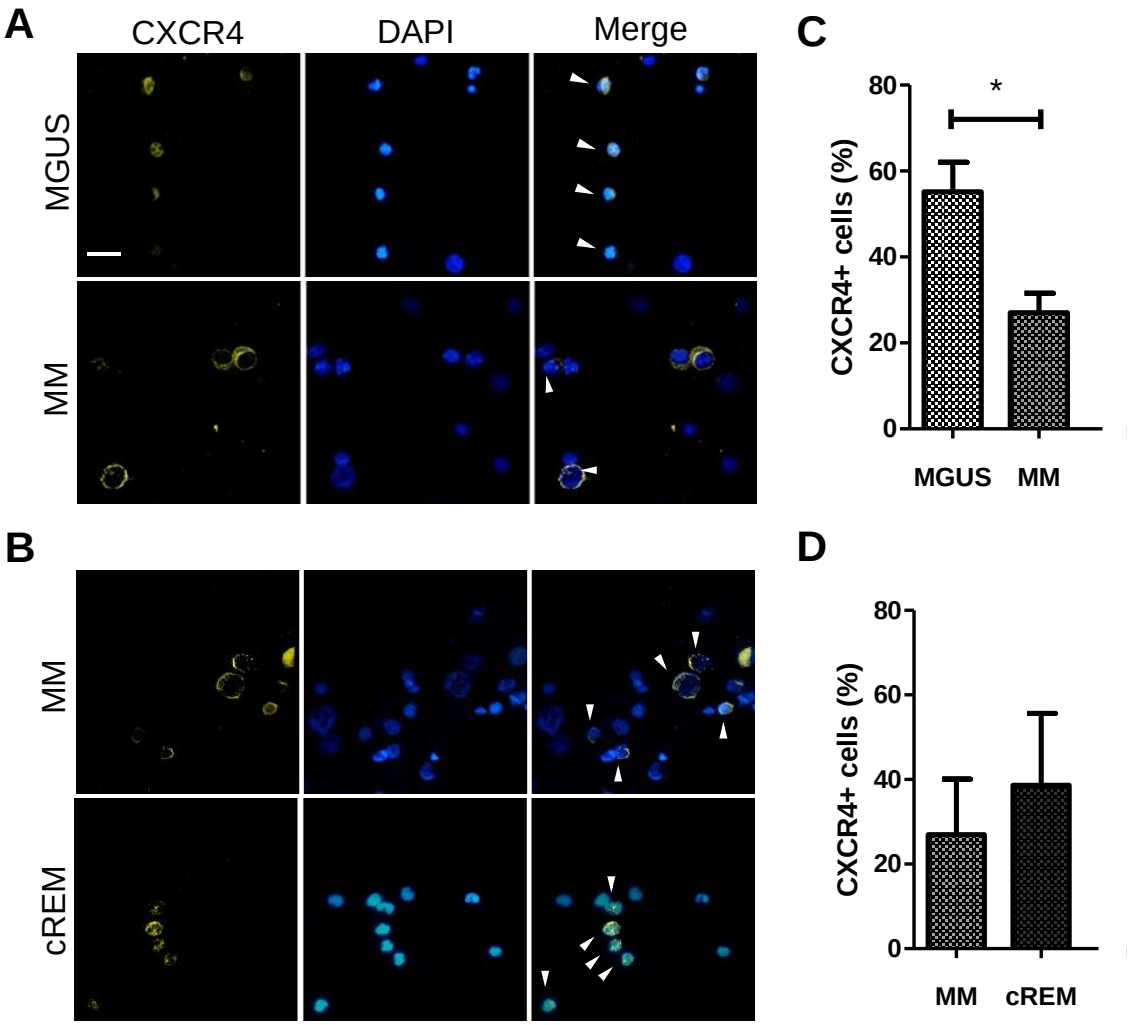

Figure 9: Bone marrow (BM) C-X-C motif chemokine receptor (CXCR)4$^+$ cells from monoclonal gammopathy of undetermined significance (MGUS), multiple myeloma (MM) and complete remission (cREM) patients. Representative images of BM CXCR4$^+$ cells, other than plasma cells, by immunofluorescence (white arrowheads) (A-B). Quantitative analysis of the percentage of CXCR4$^+$ cells in sequential samples of MGUS patients that evolved to MM with PCs>20% (n=3) (C), and of MM patients that achieved cREM (n=7) (D). Data is shown as mean + standard deviation, *p<0.05. Scale bar: 20 µm.

4.4. Alterations in PDGFR-β$^+$ cells percentage from MGUS to MM and from MM to cREM

The recruitment of pericytes and pericyte progenitor cells is stimulated by the ligand PDGF-B that interacts with its receptor PDGFR-β on the mentioned cells (Sá-Pereira et al. 2012). In the same manner, PDGF-B can also stimulate the recruitment of PDGFR-β$^+$ EPCs (Guo et al. 2012). Thus, PDGFR-β can be connected with EPCs recruitment in MM. Similarly to CXCR4 evaluation, we performed immunofluorescence analysis of PDGFR-β$^+$ cells in BM smears (Fig. 10A-B) and determined the % of positive cells, other than PCs, to establish the differences between the studied groups (Fig. 10C-

D) and understand if they are similar to those observed for EPCs. In MGUS, the PDGFR-β^+ cells levels were similar to those in MM, with 36.9% and 35.2%, respectively (Fig. 10C). Regarding the comparison of PDGFR-β^+ cells in MM and in cREM, the levels were significantly lower in MM than in cREM, with a progression from 14.5% to 39.0% ($p<0.001$, Fig. 10D). Thus, it is interesting to point out that the MM-cREM evolution of PDGFR-β^+ cells resembled that of EPCs in these stages.

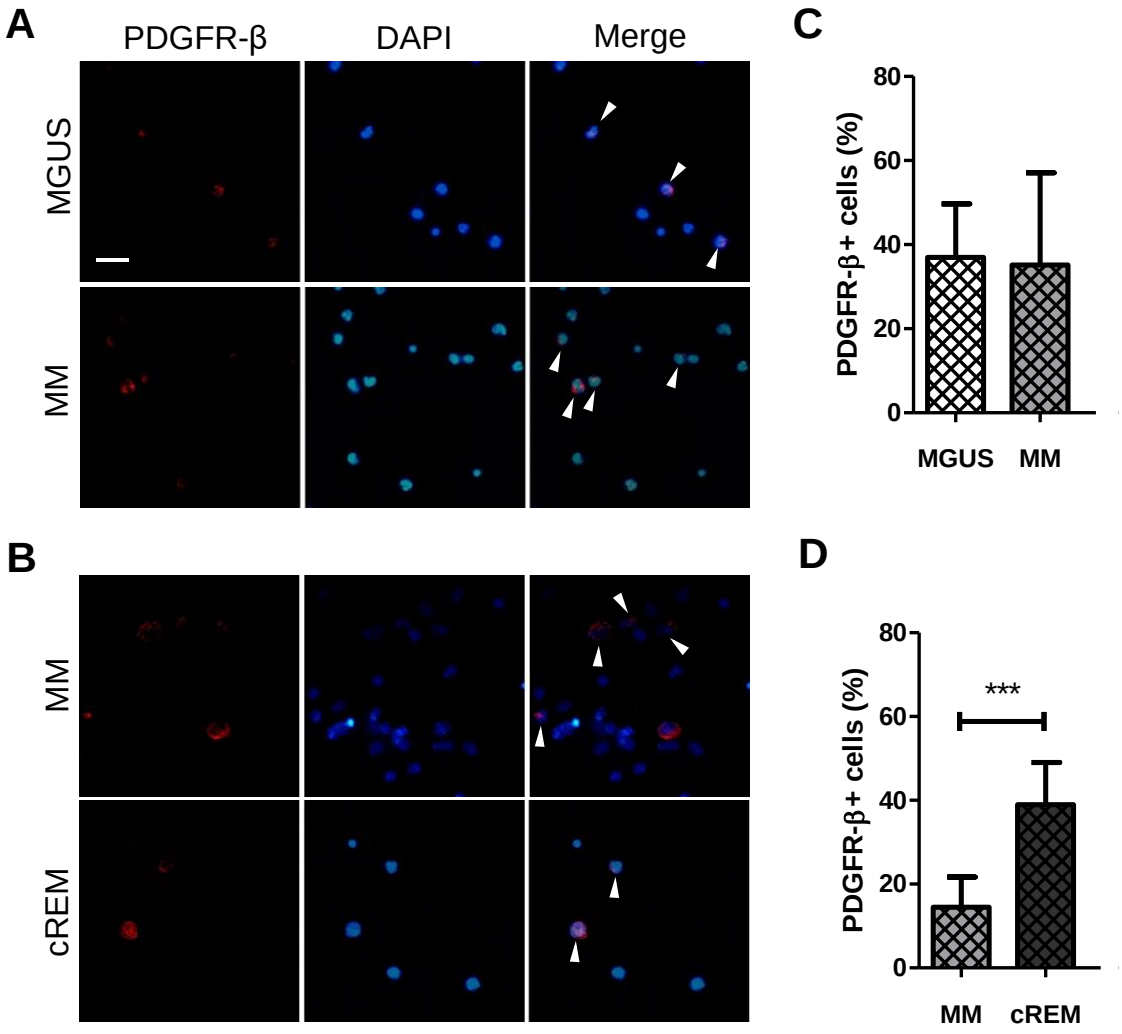

Figure 10: Bone marrow (BM) platelet-derived growth factor receptor (PDGFR)-β^+ cells from monoclonal gammopathy of undetermined significance (MGUS), multiple myeloma (MM) and complete remission (cREM) patients**.** Representative images of BM PDGFR-β^+ cells, other than plasma cells, by immunofluorescence (white arrowheads) (A-B). Quantitative analysis of the percentage of PDGFR-β^+ cells in sequential samples of MGUS patients that evolved to MM (n=3) (C), and MM patients that achieved cREM (n=8) (D). Data is shown as mean + standard deviation, ***$p<0.001$. Scale bar: 20 µm.

4.5. Correlation between plasma cells, endothelial progenitor cells, CXCR4$^+$ and PDGFR-β$^+$ cells levels from MGUS to MM and from MM to cREM

Once established the profile of cellular and molecular changes in the course of the progression of MGUS to MM, we wanted to establish if the levels of EPCs were correlated with those of PCs, which would point to EPCs as an additional biomarker of MM, and if there was a relationship between EPCs and the cells expressing CXCR4 and PDGFR-β. To this end, we analysed the results obtained in MM patients as fold change from those of the same patients in MGUS and determined the correlation coefficients between EPCs and PCs, as well as with CXCR4 and PDGFR-β positive cells. As shown in Figure 11, PCs were 4.0-fold higher in MM than in MGUS, whereas the levels of EPCs, CXCR4$^+$ cells and PDGFR-β$^+$ were nearly 0.8-fold to those of MGUS. Analysis of the correlation coefficients revealed that PCs are negatively correlated with EPCs, a finding that was observed in two groups of studied patients ($r=-0.825$, $p<0.05$, Fig. 11A and $r=-0.997$, $p<0.05$, Fig. 11B). Such analysis also showed that PCs correlated negatively with CXCR4$^+$ cells ($r=-0.825$; $p<0.05$), and that EPCs correlated positively with CXCR4$^+$ cells ($r=0.946$; $p<0.01$) (Fig. 11A), whereas no significant correlation was observed concerning PDGFR-β$^+$ cells (Fig. 11B). The significant correlations between PCs and EPCs suggest that EPCs can constitute a biomarker for MGUS progression to MM. Lastly, we also wanted to see if there was any correlation between CXCR4$^+$ and PDGFR-β$^+$ cells, but none was found (not shown), which may result from the reduced number of patients of this group (n=3).

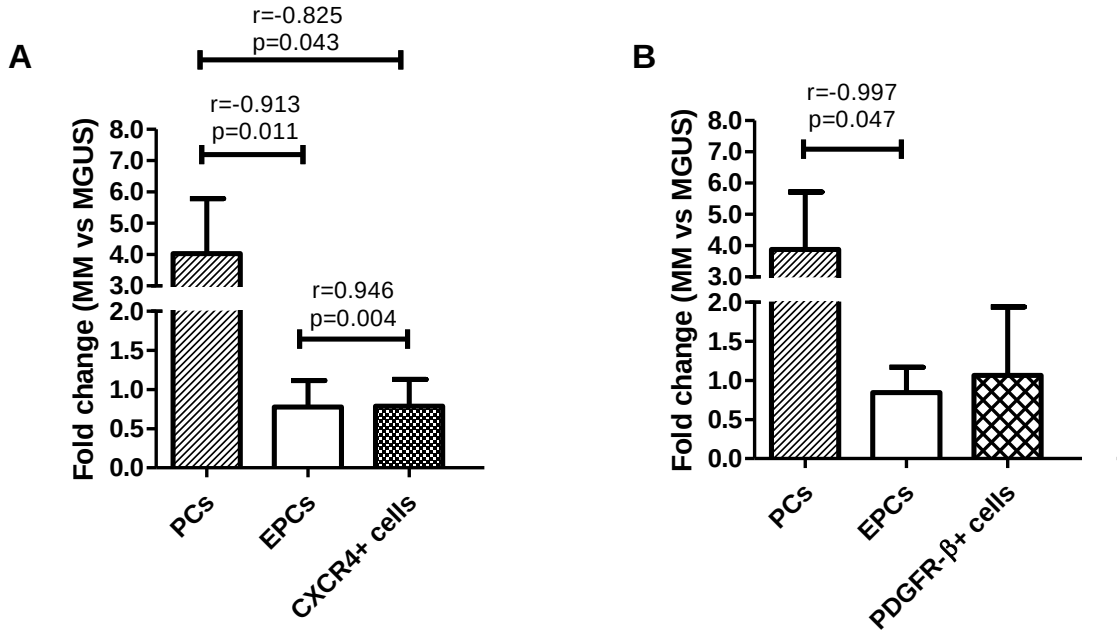

Figure 11: Changes in the levels of plasma cells (PCs), endothelial progenitor cells (EPCs), C-X-C motif chemokine receptor (CXCR)4$^+$ cells and platelet-derived growth factor receptor (PDGFR)-β$^+$ cells in multiple myeloma (MM) versus monoclonal gammopathy of undetermined significance (MGUS) and correlation coefficients between the studied parameters. Data from sequential bone marrow samples of MGUS patients that evolved to MM, concerning PCs, EPCs and CXCR4$^+$ cells (n=6) (A), and PCs, EPCs and PDGFR-β$^+$ cells (n=3) (B). Data is shown as fold change + standard deviation, correlation coefficients (r).

Similarly, we proceeded to analyse the correlation coefficients regarding the population of MM patients that achieved cREM after treatment. We observed that PCs were 0.04 to 0.05-fold lower and that EPCs levels were 1.9 to 2.7-fold higher in cREM patients as compared to MM. On the other hand, CXCR4$^+$ cells showed a 1.8-fold increase and PDGFR-β$^+$ cells had a 3.6-fold increase (Fig. 12). Concerning the correlation coefficients, none was found between PCs and EPCs levels from MM to cREM (Fig. 12A). We also evaluated the correlations between EPCs and PCs in patients who achieved pREM and non-REM, but no correlations were found as well (not shown). In the cREM group, EPCs correlated positively with both CXCR4$^+$ ($r=0.775$; $p<0.05$, Fig. 12B) and PDGFR-β$^+$ cells ($r=0.751$; $p<0.05$, Fig. 12C). Hence, the increase of EPCs amount is followed by the elevation of the expression of both CXCR4 and PDGFR-β.

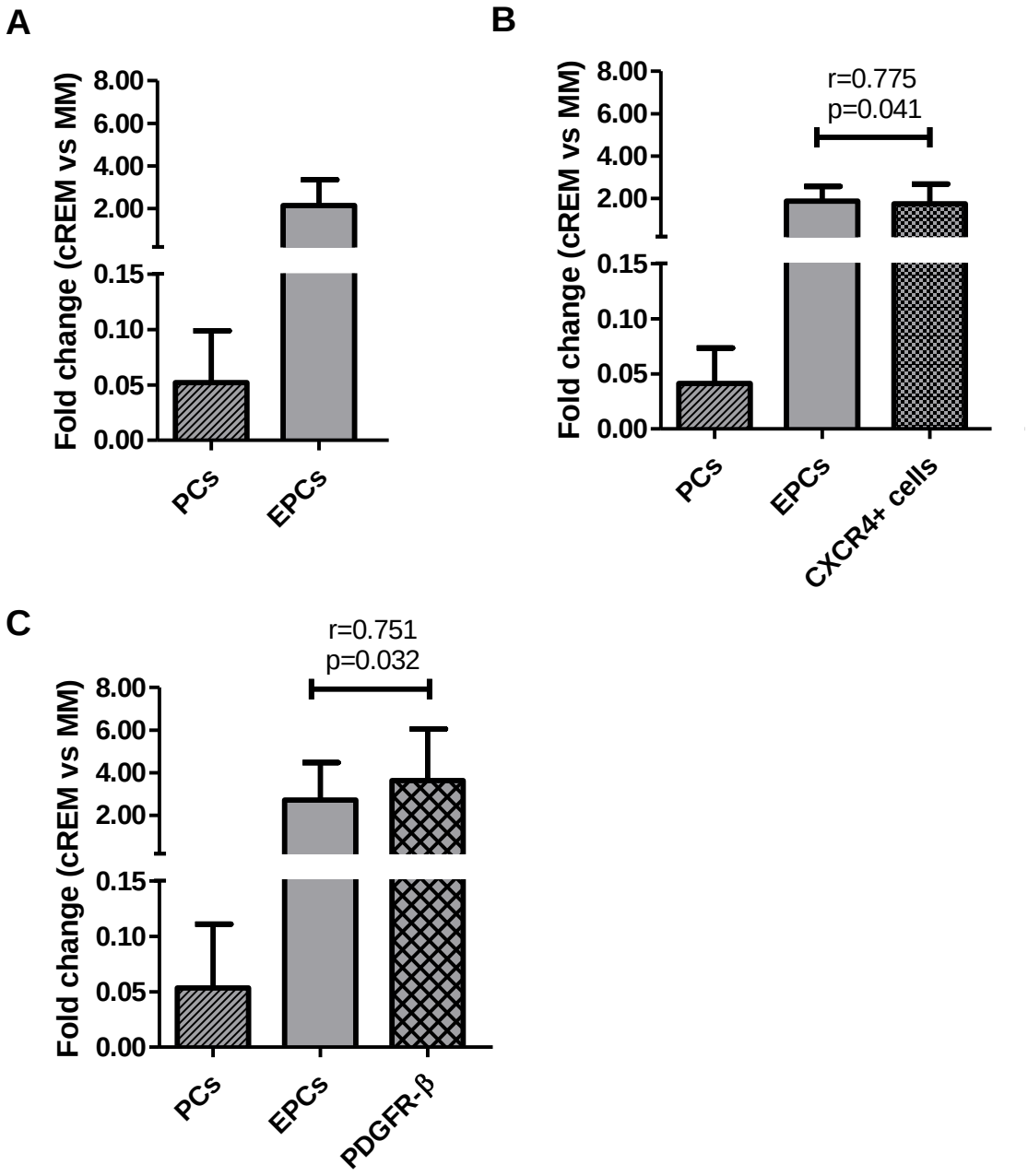

Figure 12: Changes in the levels of plasma cells (PCs), endothelial progenitor cells (EPCs), C-X-C motif chemokine receptor (CXCR)4$^+$ cells and platelet-derived growth factor receptor (PDGFR)-β$^+$ cells in complete remission (cREM) versus multiple myeloma (MM) and correlation coefficients between the studied parameters. Data from sequential bone marrow samples of MM patients that received treatment and achieved cREM, concerning PCs and EPCs (n=23) (A), PCs, EPCs and CXCR4$^+$ cells (n=7) (B) and PCs, EPCs and PDGFR-β$^+$ cells (n=8) (C). Data is shown as fold change + standard deviation, correlation coefficients (r).

5. Discussion

MM represents one of the most common haematological malignancies worldwide. Despite the diverse amount of therapies available, it remains as an incurable disease (Rajkumar 2014). The clonal expansion of malignant PCs characteristic of MM relies greatly on the development of the vasculature, of which angiogenesis has been studied (Giuliani et al. 2011). However, vasculogenesis and its key players – EPCs – remain less explored. EPCs are progenitor cells produced in the BM that incorporate new blood vessels and differentiate into ECs (Tenreiro et al. 2016). These progenitors are mobilized to the tumour site to aid in the development of the vasculature (Moschetta et al. 2016). Although the discussion of EPCs in MM is prominently increasing, conclusive results are yet to be established. More specifically, there is lack of defined levels of these cells in the BM in the several stages of MM development and after therapy, as well as of the levels of signalling receptors CXCR4 and PDGFR-β involved in events such as the recruitment of EPCs to the tumour location in MM. Assessing the levels of EPCs and the receptors mentioned can provide not only new but more sensitive markers for disease progression. Thus, we aimed at establishing the levels of BM EPCs, CXCR4 and PDGFR-β at different phases of MM, ranging from the pre-malignant stage to that achieved after treatment, in an attempt to understand how these receptors vary in the pathogenesis of MM and response to therapy.

The initially designed study included the longitudinal analysis of 30 patients in four clinical conditions, namely MGUS, MM, MM in REM and relapse, as well as the analysis of EPCs, CXCR4 and PDGFR-β in each sample. However, the reduced number of samples and BM smears per patient in each clinical condition that were provided, hampered with the realization of the study as outlined and undermined the statistical significance of our results. Nonetheless, to the best of our knowledge, this is the first retrospective and sequential study evaluating EPCs levels in patients evolving from MGUS to MM, as well as from MM to treated MM, distinguishing the pattern of EPCs accordingly with the stage of REM or no REM achieved, and correlating the data with the expression of the signalling molecules CXCR4 and PDGFR-β, in addition to the widely used marker of MM, PCs.

The first challenge of the present project was to develop a method to identify and quantify EPCs in archived BM smears. Although flow cytometry is the most chosen technique to quantify and characterize EPCs in MM (Braunstein et al. 2006, Udi et al. 2011, Moschetta et al. 2016), it can only be applied to archived frozen BM samples and not to archived BM smears. Hence, we had to turn to immunofluorescence to evaluate the archived BM smears. Due to the absence of a specific marker of EPC, we selected

three of the recognized markers of this cellular population and made use of laser confocal microscopy to perform triple labelling immunofluorescence analysis, together with nuclear labelling with DAPI. While we observed the percentage of EPCs as >10%, authors who evaluated EPC content through flow cytometry obtained a much lower percentage of EPCs (<1%). This is probably due the largest cellular population – erythrocytes – being included in the cell count. Furthermore, several studies regarding *in vitro* culture of putative EPCs have described several phenotypes, such as cerioid, cobblestone-appearance, spindle shaped and round cells (Gehling et al. 2000, Eggermann et al. 2003, Sun et al. 2014). We observed EPCs as round shaped cells. Hence, different methodologies produce different results. Indeed, comparisons of our observations with published studies are challenging due to differences in patients number and characteristics, and different techniques and markers used to evaluate EPC content. The principal advantage of our method consists on the fact that it can be applied to archived BM smears, allowing for a retrospective analysis of patients. In this context, this methodology was used to study MM, but it can also be used for other pathologies involving enhanced blood vessel development in the BM. Thus, we developed a methodology that generated multiple-colour immunofluorescence images to detect EPCs in archived BM smears. This method is an accessible approach to monitor vasculogenesis through the analysis of EPCs, which can be implemented in any laboratory equipped with a suitable microscope.

Regarding EPCs levels, Moschetta *et al*. recently observed that BM $CD34^+VEGFR\text{-}2^+$ EPCs levels were slightly higher in SMM than in MM, but it failed to reach statistical significance (Moschetta et al. 2016). On the other hand, several studies demonstrated that cEPCs levels are higher in MM in comparison to MGUS and healthy controls (Bhaskar et al. 2012, Moschetta et al. 2016). Since the BM constitutes the natural source of EPCs, we speculate that for EPCs to go into circulation in MM, they must exist in higher levels in the BM in previous stages such as MGUS. Notably, we observed that MGUS patients who evolve to MM with BM PCs>20% displayed significantly higher levels of BM EPCs in MGUS, than those who evolve to MM with BM PCs≤20%. Therefore, EPCs in MGUS can constitute a biomarker for worse progression of MM. Opposed to our observation, Udi *et al*. reported that MM patients with BM-infiltration rates≥20% have a slightly higher content of BM $CD34^+CD133^+VEGFR\text{-}2^+$ EPCs in MM in comparison to MGUS patients, but it also failed to achieve significance (Udi et al. 2011). Furthermore, we observed a statistically significant negative correlation between EPCs and PCs in the group of patients that evolved from MGUS to MM, indicating that while the % of PCs grows, that of EPCs decreases. Thus, we hypothesize

that during MM, when PCs are predominant, EPCs have already started integrating and differentiating into ECs in the new blood vessels, which explains the lower BM EPCs levels in MM. By definition, angiogenesis is the process that completes vasculogenesis. Hence, vasculogenesis may start being stimulated in MGUS, while angiogenesis is known to be predominant in MM (Lee et al. 2015). The transition of MGUS to MM is known to be highly stimulated by an "angiogenic switch", which is characterized by an overflow of angiogenic cytokines by MM cells or other cells from the tumour microenvironment (Otjacques et al. 2011). Outstandingly, Moschetta and colleagues observed that the angiogenic dependency happens during SMM and that the mobilization of EPCs occurs early on MM (Moschetta et al. 2016), which further highlights the importance of EPCs during initial stages of MM. On the topic of response to treatment, cEPCs and BM $CD34^+VEGFR\text{-}2^+$ ECPs levels were lower after treatment, whereas BM $CD34^+CD133^+VEGFR\text{-}2^+$ EPCs displayed no significant difference (Zhang et al. 2005, Udi et al. 2011, Bhaskar et al. 2012). On the contrary, our results showed that BM EPCs levels rise from MM to REM, and that patients that have a poor response to treatment display in MM higher EPCs than the ones who achieve REM. This discrepancy with the literature may be due to different applied methodology such as flow cytometry to analyse EPCs content and to the fact that the studies mentioned used different patients in different MM phases. We could not find a correlation between EPCs levels and PCs in the patients with MM that received treatment. This may be due to the diversity of treatments applied, considering that besides targeting PCs, several drugs such as thalidomide, lenalidomide, bortezomib and dexamethasone also have direct or indirect anti-angiogenic properties (Wang et al. 2015). Nevertheless, our results show that EPCs can constitute a possible biomarker for response to therapy, since MM EPCs levels were increased in patients that achieved REM, but remained unchanged in patients who did not.

EPCs are mobilized from one area in the BM to the MM PCs site where they will incorporate the vasculature and differentiate into ECs (Moschetta et al. 2016). This mobilization is greatly influenced by a chemotactic gradient established by CXCL12 and exerted on cells expressing its receptor CXCR4. In the BM, several cells such as ECs secrete CXCL12 and stimulate the mobilization of MM PCs (Menu et al. 2006). On the other hand, EPCs express CXCR4 (Lu et al. 2015), so CXCL12 may also participate in EPCs mobilization. The CXCR4/CXCL12 axis has also been demonstrated to stimulate proliferation and differentiation of progenitor cells into ECs (Li et al. 2015). Although there are studies evaluating the temporal evolution of CXCL12 from MGUS to MM (Martin et al. 2006), to the best of our knowledge, none has yet evaluated the temporal evolution

of CXCR4, nor its connection to EPCs, in MM. Therefore, we analysed the levels of $CXCR4^+$ cells throughout MM development. In order to distinguish $CXCR4^+$ cells from MM PCs also expressing the receptor, we only counted the cells whose morphology did not resemble PCs. Our results show that the amount of cells expressing CXCR4 is higher in MGUS in comparison to MM and, similarly to EPCs, $CXCR4^+$ cells were also significantly higher in MGUS in patients who evolved to MM with higher BM infiltration than the patients with lower BM PCs. On the other hand, while analysing the full group of MGUS patients who evolved to MM, there was a positive correlation between EPCs and $CXCR4^+$ cells, which may result from an increased expression of the receptor as EPCs levels raise. Furthermore, its ligand CXCL12 was connected to the upregulation of MMP-9, which aids in the release of EPCs from their stem cell niche (Heissig et al. 2002). Considering that EPCs may have already started incorporating into blood vessels in MM, it is plausible that the release of EPCs from their niche and recruitment starts in MGUS, while their differentiation is more characteristic of MM. Despite EPCs correlating positively with $CXCR4^+$ cells in the group of patients studied from MM to cREM, no significant differences were found between MM and cREM CXCR4 expression levels, which is conceivable due to different treatments applied. Nonetheless, higher CXCR4 levels in cREM may be related with the stimulation of EPCs proliferation after therapy. Thus, these data point to the evolution of EPCs levels being correlated to CXCR4 levels and that the variation in the levels of $CXCR4^+$ cells in different disease stages may be related with significant events in which the receptor is involved, such as cell recruitment, differentiation and proliferation.

PDGFR-β and its ligand PDGF-B are known to participate in vascular growth and recruitment of pericytes that contribute to the stabilization of the microvasculature (Sá-Pereira et al. 2012). During blood vessel formation, pericytes must detach from the vessels to allow migration and proliferation of ECs. After this process is completed, pericytes are recruited to re-establish vessel stabilization (Papetti and Herman 2002). While PDGF-B is secreted by ECs, its receptor PDGFR-β is expressed on pericytes (Sá-Pereira et al. 2012). Additionally, EPCs express PDGFR-β (Guo et al. 2012), which overexpression is connected with a higher re-endothelialisation ability (Wang et al. 2014). MM PCs can also express PDGFR-β, which is correlated with proliferation and migration. Furthermore, this expression of PDGFR-β by PCs seems to be increased in MM in comparison to MGUS (Coluccia et al. 2008). Since the PDGFR-β/PDGF-B axis can induce proliferation and migration of MM PCs and pericytes, it is conceivable that it may also have this effect on EPCs. With attention to MM PCs, we again excluded the cells with their characteristic morphology from the count of PDGFR-β^+ cells. Along the

transition from MGUS to MM, we did not observe changes in the % of PDGFR-β^+ cells, nor any significant correlation with EPCs, in the reduced population of 3 patients analysed. On the subject of therapy, EPCs levels were positively correlated with PDGFR-β^+ cells. Hence, EPCs may express PDGFR-β and this receptor may play an important part in the migration and proliferation of EPCs in MM. Moreover, we observed that CXCR4$^+$ and PDGFR-β^+ cells levels were similar in these phases. Although we could not check for a correlation between these levels since the analysis was made on different groups of patients, these receptors may be connected.

To sum up, our findings hint to the potential of BM EPCs as a biomarker for both MM progression and response to therapy, since higher levels of BM EPCs in MGUS and MM seem to be connected with a worsen progression of MM and with a worse response to therapy, respectively. This may also justify more studies focusing on therapy directed to EPCs in MM patients, as increased levels seem to contribute to disease progression. Furthermore, there is the need to explore the molecules and the respective receptors involved in the recruitment, proliferation and differentiation of EPCs as the expression of CXCR4 and PDGFR-β seem to be associated with EPCs in MM.

6. Conclusions and future perspectives

The knowledge concerning EPCs and how these cells participate in the development of blood vessels, whether for beneficial purposes such as tissue repair or in harmful scenarios like malignancies, has grown immensely ever since EPCs were first mentioned. However, despite the attention given to EPCs on MM in the last decade, it remains mostly an unexplored territory. Our study has shown that BM EPCs levels vary amongst different phases of MM. Moreover, elevated levels of EPCs in MGUS and before therapy administration show that EPCs can be a good biomarker for disease progression and response to therapy. Since the biggest controversy associated with EPCs is their identification, exploring several combinations of EPCs markers simultaneously would be of relevance, while performing assays to determine their proliferative potential and differentiation into ECs. The additional use of CD45 to our combination of EPC markers would be important, since it allows for the exclusion of HSCs that do not constitute true EPCs and express CD45. For example, in order to better understand the differentiation of EPCs, EPCs could be isolated from mouse BM, marked with a fluorescent probe and re-inserted into the BM. This would allow for the observation of EPCs differentiation pattern by multiple BM extractions throughout MM development. Regarding treatment, it would also be of value to develop a therapy targeting EPCs. Although the relation between thalidomide, lenalidomide, bortezomib and stem cell transplant and EPCs levels in the BM and PB has been explored, there is lack of an uniformed study incorporating the high number of therapies available and relating them to EPCs levels.

Our results suggest that, from the asymptomatic stage to the symptomatic and after treatment administration, the evolution of EPCs levels is followed by a similar evolution of both CXCR4 and PDGFR-β. In order to complete the present study, the temporal changes of these receptors should be studied simultaneously with the respective ligands, CXCL12 and PDGF-B. In addition, due to PCs being able to express the receptors and ligands, PCs should be marked with a specific PC marker such as CD138 to allow for a proper identification. On the other hand, there is also the need to assess which EPC-related process is more pronounced in each MM phase such as recruitment, mobilization, adhesion, proliferation and differentiation. For instance, in order to verify if the cells expressing CXCR4 or PDGFR-β are proliferating, co-staining of CXCR4 or PDGFR-β with the proliferation marker Ki-67 could be performed. Furthermore, it was shown that PDGF-BB was able to stimulate the differentiation of EPCs into smooth muscle cells, for which it would of value to study if this differentiation is stimulated in MM. Lastly, the clarification the mechanisms related to EPCs could offer

novel targets to modulate the vasculature development and therefore, the progression of MM.

A deeper understanding of the time-course of both EPCs and key molecular players in EPCs is pivotal to develop treatment adapted to the temporal evolution of MM. Moreover, it would provide novel targets to modulate vascular dysfunction and prevent or delay events such as the progression of MGUS to MM and promote a more personalized therapy.

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8. Annexes

Supplementary Table 1: Experimental conditions tested during protocol optimization of multiple labelling of CD34, CD133 and VEGFR-2

Antibody	Fixation	Permeabilization	Blocking	Primary Antibody	Secondary Antibody	DAPI
CD34	100% Methanol, 1 h, 4ºC	0.5% Triton X-100 in PBS, 20 min, RT	3% BSA in PBS, 1 h, RT	1:100, ON, 4ºC	1:500, 1 hour, RT	1:1000, 2 min, RT
	100% Methanol, 1 h, 4ºC	0.5% Triton X-100 in PBS, 20 min, RT	3% BSA in PBS, 1 h, RT	1:200, ON, 4ºC	1:500, 1 hour, RT	1:1000, 2 min, RT
	2.5% Glutaraldehyde, 1 h, RT	0.5% Triton X-100 in PBS, 20 min, RT	3% BSA in PBS, 1 h, RT	1:100, ON, 4ºC	1:250, 1 hour, RT	1:1500, 2 min, RT
	2.5% Glutaraldehyde, 1 h, RT	0.5% Triton X-100 in PBS, 20 min, RT	3% BSA in PBS, 1 h, RT	1:100, 48 h, 4ºC	1:500, 1 hour, RT	1:1500, 2 min, RT
	1% Paraformaldehyde, 5 min, RT	0.5% Triton X-100 in PBS, 20 min, RT	3% BSA in PBS, 1 h, RT	1:100, ON, 4ºC	1:250, 1 hour, RT	1:1500, 2 min, RT
	50% Methanol/50% Acetone, 30 min, -20ºC	0.5% Triton X-100 in PBS, 20 min, RT	3% BSA in PBS, 1 h, RT	1:100, ON, 4ºC	1:250, 1 hour, RT	1:1500, 2 min, RT
	50% Methanol/50% Acetone, 10 min, -20ºC	0.5% Triton X-100 in PBS, 20 min, RT	3% BSA in PBS, 1 h, RT	1:100, ON, 4ºC	1:250, 1 hour, RT	1:1500, 2 min, RT
	50% Ethanol/50% Acetone, 10 min, -20ºC	0.5% Triton X-100 in PBS, 20 min, RT	3% BSA in PBS, 1 h, RT	1:100, ON, 4ºC	1:250, 1 hour, RT	1:1500, 2 min, RT
	100% Acetone, 10 min, -20ºC	0.5% Triton X-100 in PBS, 20 min, RT	3% BSA in PBS, 1 h, RT	1:100, ON, 4ºC	1:250, 1 hour, RT	1:1500, 2 min, RT
	50% Methanol/50% Acetone, 20 min, -20ºC	0.5% Triton X-100 in PBS, 20 min, RT	3% BSA in PBS, 1 h, RT	1:100, ON, 4ºC	1:250, 1 hour, RT	1:1500, 2 min, RT
	75% Methanol/25% Acetone, 20 min, -20ºC	0.5% Triton X-100 in PBS, 20 min, RT	3% BSA in PBS, 1 h, RT	1:100, ON, 4ºC	1:250, 1 hour, RT	1:1500, 2 min, RT
	75% Methanol/25% Acetone, 20 min, -20ºC		3% BSA in PBS, 1 h, RT	1:100, ON, 4ºC	1:250, 1 hour, RT	1:1500, 2 min, RT
	75% Methanol/25% Acetone/0.01% Triton X-100, 20 min, -20ºC		3% BSA in PBS, 1 h, RT	1:100, ON, 4ºC	1:250, 1 hour, RT	1:1500, 2 min, RT
	75% Methanol/25% Acetone/0.05% Triton X-100, 20 min, -20ºC		3% BSA in PBS, 1 h, RT	1:100, ON, 4ºC	1:250, 1 hour, RT	1:1500, 2 min, RT
	75% Methanol/25% Acetone/0.01% Triton X-100, 20 min, -20ºC		10% RS and 1% BSA in PBS, 1 h, RT	1:100, ON, 4ºC	1:250, 1 hour, RT	1:1500, 2 min, RT
	75% Methanol/25% Acetone/0.05% Triton X-100, 20 min, -20ºC		10% RS and 1% BSA in PBS, 1 h, RT	1:100, ON, 4ºC	1:250, 1 hour, RT	1:1500, 2 min, RT
	75% Methanol/25% Acetone/0.01% Triton X-100, 20 min, -20ºC		5% RS and 1% BSA in PBS, 1 h, RT	1:100, ON, 4ºC	1:250, 1 hour, RT	1:1500, 2 min, RT
	75% Methanol/25% Acetone/0.05% Triton X-100, 20 min, -20ºC		5% RS and 1% BSA in PBS, 1 h, RT	1:100, ON, 4ºC	1:250, 1 hour, RT	1:1500, 2 min, RT

	75% Methanol/25% Acetone/0.01% Triton X-100, 20 min, -20ºC		10% RS and 1% BSA in PBS, 1 h, RT	1:100, ON, 4ºC	1:250, 1 hour, RT	1:1000, 3 min, RT
VEGFR-2	100% Methanol, 1 h, 4ºC	0.5% Triton X-100 in PBS, 20 min, RT	3% BSA in PBS, 1 h, RT	1:200, ON, 4ºC	1:500, 1 hour, RT	1:1000, 5 min, RT
	100% Methanol, 1 h, 4ºC	0.5% Triton X-100 in PBS, 20 min, RT	3% BSA in PBS, 1 h, RT	1:1000, ON, 4ºC	1:500, 1 hour, RT	1:1000, 5 min, RT
	2.5% Glutaraldehyde, 1 h, RT	0.5% Triton X-100 in PBS, 20 min, RT	3% BSA in PBS, 1 h, RT	1:1000, ON, 4ºC	1:500, 1 hour, RT	1:1500, 2 min, RT
	2.5% Glutaraldehyde, 1 h, RT	0.5% Triton X-100 in PBS, 20 min, RT	3% BSA in PBS, 1 h, RT	1:1000, ON, 4ºC	1:1000, 1 hour, RT	1:1500, 2 min, RT
	1% Paraformaldehyde, 5 min, RT	0.5% Triton X-100 in PBS, 20 min, RT	3% BSA in PBS, 1 h, RT	1:1000, ON, 4ºC	1:500, 1 hour, RT	1:1500, 2 min, RT
	50% Methanol/50% Acetone, 30 min, -20ºC	0.5% Triton X-100 in PBS, 20 min, RT	3% BSA in PBS, 1 h, RT	1:1000, ON, 4ºC	1:500, 1 hour, RT	1:1500, 2 min, RT
	50% Methanol/50% Acetone, 10 min, -20ºC	0.5% Triton X-100 in PBS, 20 min, RT	3% BSA in PBS, 1 h, RT	1:1000, ON, 4ºC	1:500, 1 hour, RT	1:1500, 2 min, RT
	50% Ethanol/50% Acetone, 10 min, -20ºC	0.5% Triton X-100 in PBS, 20 min, RT	3% BSA in PBS, 1 h, RT	1:1000, ON, 4ºC	1:500, 1 hour, RT	1:1500, 2 min, RT
	100% Acetone, 10 min, -20ºC	0.5% Triton X-100 in PBS, 20 min, RT	3% BSA in PBS, 1 h, RT	1:1000, ON, 4ºC	1:500, 1 hour, RT	1:1500, 2 min, RT
	75% Methanol/25% Acetone/0.01% Triton X-100, 20 min, -20ºC		10% GS and 1% BSA in PBS	1:200, ON, 4ºC	1:500, 1 hour, RT	1:1500, 2 min, RT
	75% Methanol/25% Acetone/0.01% Triton X-100, 20 min, -20ºC		10% GS and 1% BSA in PBS	1:500, ON, 4ºC	1:500, 1 hour, RT	1:1500, 2 min, RT
CD133	75% Methanol/25% Acetone/0.01% Triton X-100, 20 min, -20ºC		10% GS and 1% BSA in PBS	1:50, ON, 4ºC	1:500, 1 hour, RT	1:1500, 2 min, RT

BSA, bovine serum albumin; CD, cluster of differentiation; DAPI, 4',6-diamidino-2-phenylindole; GS, goat serum; ON, overnight; PBS, phosphate buffered saline; RS, rabbit serum; RT, room temperature; VEGFR-2, vascular endothelial growth factor receptor.

Supplementary Table 2: Tested combinations of the antibodies CD34, CD133 and VEGFR-2

First BS	Primary antibodies	Secondary antibodies	Second BS	Primary antibody	Secondary antibody	Third BS	Primary antibody	Secondary antibody
10% GS and BSA 1% in PBS	CD133 (1:50) + VEGFR-2 (1:500) in the first BS	Alexa Fluor® 555 (1:500) + Alexa Fluor® 647 (1:500) in the first BS	10% RS and BSA 1% in PBS	CD34 (1:100) in the second BS	Alexa Fluor® 488 (1:250) in the second BS	-	-	-
10% GS and BSA 1% in PBS	CD133 (1:50) + VEGFR-2 (1:500) in the first BS	Alexa Fluor® 555 (1:500) + Alexa Fluor® 647 (1:500) in the first BS	-	-	-	-	-	-
10% GS and BSA 1% in PBS	VEGFR-2 (1:500) in the first BS	Alexa Fluor® 647 (1:500) in the first BS	10% RS and BSA 1% in PBS	CD34 (1:100) in the second BS	Alexa Fluor® 488 (1:250) in the second BS	-	-	-
10% GS and BSA 1% in PBS	CD133 (1:100) in the first BS	Alexa Fluor® 555 (1:500)v in the first BS	10% RS and BSA 1% in PBS	CD34 (1:100) in the second BS	Alexa Fluor® 488 (1:250) in the second BS	10% GS and BSA 1% in 1X PBS	VEGFR-2 (1:200) in the third BS	Alexa Fluor® 647 (1:500) in the third BS
10% GS and BSA 1% in PBS	CD133 (1:100) + VEGFR-2 (1:200) + CD34 (1:100) in the first BS	Alexa Fluor® 555 (1:500) + Alexa Fluor® 647 (1:500) + Alexa Fluor® 488 (1:250) in the first BS	-	-	-	-	-	-
10% GS and BSA 1% in PBS	CD133 (1:100) + VEGFR-2 (1:100) in the first BS	Alexa Fluor® 555 (1:500) + Alexa Fluor® 647 (1:500) in the first BS	10% RS and BSA 1% in PBS	CD34 (1:100) in the second BS	Alexa Fluor® 488 (1:250) in the second BS	-	-	-
10% GS and BSA 1% in PBS	CD133 (1:100) + VEGFR-2 (1:200) in the first BS	Alexa Fluor® 555 (1:500) + Alexa Fluor® 647 (1:500) in the first BS	10% RS and BSA 1% in PBS	CD34 (1:100) in the second BS	Alexa Fluor® 488 (1:250) in the second BS	-	-	-

BS, blocking solution; CD, cluster of differentiation; GS, goat serum; PBS, phosphate buffered saline; RS, rabbit serum; VEGFR-2, vascular endothelial growth factor receptor 2.